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A AND B GENES IN THE TOMATO *L. ESCULENTUM*
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TRANSFER AND EXPRESSION OF THE *BACILLUS*
BRANCHED-CHAIN α -OXO ACID DECARBOXYLASE A AND B GENES
IN THE TOMATO *L. ESCULENTUM* VAR. ROMA

BY



GEFU WANG

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND
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FACULTY OF GRADUATE STUDIES AND RESEARCH

THE UNDERSIGNED CERTIFY THAT THEY HAVE READ, AND RECOMMEND TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH FOR ACCEPTANCE, A THESIS ENTITLED **TRANSFER AND EXPRESSION OF THE *BACILLUS* BRANCHED-CHAIN α -OXO ACID DECARBOXYLASE A AND B GENES IN THE TOMATO *L. ESCULENTUM* VAR. ROMA** SUBMITTED BY **GEFU WANG** IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF **DOCTOR OF PHILOSOPHY IN PLANT MOLECULAR BIOLOGY.**

TO MY PARENTS AND MY SON

ABSTRACT

Engineering of higher plants for increased cold tolerance will require the chemical modification of membrane fluidity in both organelles and in the cytoplasm of plants. Membrane fluidity is mainly dependent on the phase transition temperature of membrane phospholipids, which is determined by the ratio of saturated and unsaturated straight chain fatty acids present in the membrane. A small number of microorganisms utilize branched-chain fatty acids (BCFA) instead of unsaturated fatty acids as their membrane constituents. Microorganisms with elevated levels of BCFA were found to grow under stress conditions such as suboptimal temperature and hydrostatic pressure. BCFA were found to be present in plants such as *Nicotiana tabacum* and *Antirrhinum majus*. One of the key enzymes facilitating synthesis of BCFA in *Bacillus subtilis* 168s is the branched-chain α -oxo acid decarboxylase (BCOADc). To determine the role of BCFA as components of plant membranes, the A and B genes encoding the α and β polypeptides of BCOADc were simultaneously introduced into tobacco and tomato plants. Expression of the A and B genes was activated by the *mas* promoters (P1 and P2) present in the plant expression vector system. Transgenic plants were regenerated and the copy number of the integrated A and B genes was determined by Southern hybridization. Analysis of the *mas* promoter driven transcripts of the A and B genes was performed by primer extension experiments. Presence of the α and β polypeptides in transgenic tomato tissues was verified by immunoblot analysis. Comparative analysis of a small number of transgenic tomato plants grown at 22°C and 4°C showed enhanced tolerance to 4°C when compared to untransformed control plants. These findings, if confirmed by a larger scale analysis, may suggest a positive role of BCFA as the protective mechanism for growth of plants under suboptimal temperatures. Analyzing tomato plants transformed with a construct containing the bacterial luciferase fusion gene *luxF* under the control of the P1 promoter resulted in a finding that the *mas* promoters function in leaf and fruit tissues of tomato plants at 4°C. This finding indicates that the *mas* promoters may be useful for expression of protective genes in response to cold shock and for further engineering of plants to be grown under suboptimal temperature conditions.

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ABBREVIATIONS

A gene:	Gene for α subunit of BCOADc
ATP:	Adenosine 5'-triphosphate
B gene:	Gene for β subunit of BCOADc
BAP:	Benzyl amino purine
BCOADc:	Branched-chain α -oxo acid decarboxylase
BCOADh:	Branched-chain α -oxo acid dehydrogenase
BSA:	Bovine serum albumin
CsCl:	Cesium chloride
CTP:	Cytidine 5'-triphosphate
dATP:	2'-Deoxyadenosine 5'-triphosphate
dCTP:	2'-Deoxycytidine 5'-triphosphate
Decanal:	Decyl aldehyde [$\text{CH}_3(\text{CH}_2)_8\text{CHO}$]
ddH ₂ O:	Double-distilled water
DEPC:	Diethyl pyrocarbonate
dGTP:	2'-Deoxyguanosine 5'-triphosphate
DMSO:	Dimethyl sulfoxide
DTT:	Dithiothreitol
dTTP:	2'-Deoxythymidine 5'-triphosphate
EDTA:	Ethylenediaminetetra acetic acid
EGTA:	Ethylene glycol-bis (β -aminoethyl ether) N, N, N', N'-tetroacetic acid
EtBr:	Ethidium bromide
FMN:	Flavin mononucleotide
FMNH ₂ :	Reduced flavin mononucleotide
g:	Gram
GTP:	Guanosine 5'-triphosphate
IPTG:	Isopropyl β -D-thiogalactopyranoside
L:	Liter
LB:	Left border sequence of T-DNA
<i>luxA</i> :	Bacterial luciferase α subunit encoding gene
<i>luxB</i> :	Bacterial luciferase β subunit encoding gene
<i>luxF</i> :	Bacterial luciferase fusion gene
<i>mas</i> :	Mannopine synthase
mg:	Milligram
ml:	Milliliter
NAA:	Naphthalene acetic acid
NaOAc:	Sodium acetate
ng:	Nanogram
NH ₄ Ac:	Ammonium acetate
NPTII:	Neomycin phosphotransferase II
OD:	Optical density
ORF:	Open reading frames
PCR:	Polymerase chain reaction

PEG:	Polyethylene glycol
PMSF:	Phenylmethanesulfonyl fluoride
RB:	Right border sequence of T-DNA
RNase:	Ribonuclease
SDS:	Sodium dodecyl sulfate
ssDNA:	Single-stranded DNA
TEMED:	N, N, N', N'-Tetramethylethylenediamine
T _m :	Phase transition temperature (melting temperature)
Tris:	Tri (hydroxy methyl) amino methane
TTP:	Thymidine 5'-triphosphate
μg:	Microgram
μl:	Microliter
UV:	ultraviolet
X-gal:	5-bromo-4-chloro-3-indolyl galactoside

INTRODUCTION

1. From Biology to Biotechnology

Tomorrow's plants can be created by today's technology. The introduction of new traits into plants no longer depends upon spontaneous mutation and selective breeding. Genetic techniques have been developed to isolate specific genes and transfer them into organisms of different species, even into organisms of different kingdoms. Many plants of the twenty-first century will be transgenic, i.e., genetically engineered to express genes foreign to the plant. C. Manly Molpus, President of the Grocery Manufacturers of America, has predicted that over 50 bioengineered foods will be in the marketplace by the year 2000 (Pfeiffer, 1994).

The successful development of transgenic plants is a labor, technology, and information intensive process. A thorough understanding of the **essential elements of plant transformation** is but the first step. Complex mechanisms for gene transfer, gene expression, and gene selection are involved. Innovative ancillary techniques also must be employed to visualize and quantitate the results of transformation. One such technique is the co-expression of a **lux reporter gene** with the foreign gene in plant cells. The luciferase gene product permits the localization and estimation of foreign gene expression in living plants throughout their development.

The results are preliminary but encouraging from **early transgenic plant studies** which have targeted (1) resistance to insect pests and (2) cold tolerance. The strategies of these early investigations have required revision and refinement to obtain significant foreign gene expression. As the **biochemistry of both the plant and the potential foreign gene donors** becomes better understood, new approaches to solving these and other problems may become apparent.

2. Plant Transformation

2.1 Essential elements of *Agrobacterium* transformation

The first element of a plant transformation system is a mechanism for transferring foreign genes into plant cells. A naturally occurring mechanism was identified in the 1970s. The pathogen *Agrobacterium tumefaciens* transfers some of its genes into plant cells to facilitate its own reproduction. The result is the formation of a crown gall tumor at the site of infection. The exploitation of *Agrobacterium* as a mediator of foreign gene transfer was made possible by the elucidation of this mechanism. Two of the three genetic components controlling *Agrobacterium* transformation were shown to be located on the Ti plasmid; the third component was located in the host genome.

The first component of the mechanism, the T-DNA of the Ti plasmid, contains the genes which enhance plant auxin and cytokine biosynthesis, producing a tumor in the process. Unlike classical transposons, T-DNA does not mediate its own transfer (Zambryski et al., 1983). The right and left border sequences (RB and LB), which flank the T-DNA will transfer any DNA, even foreign DNA placed between them (Hemalsteens et al., 1980).

The second component of the mechanism, the virulence (*vir*) region of the Ti plasmid, provides most of the trans-acting products for T-DNA mobilization and transfer to the plant cell. Although the *vir* products are produced on the same plasmid as the T-DNA, because they are trans-acting, their genes do not necessarily need to be located on the same plasmid as the T-DNA to be transferred (Hoekema et al., 1983). The 30 Kb *vir* region is organized into six complementation groups: four genes (*virA*, *virB*, *virD*, and *virG*) are essential for plant cell transformation and two genes (*virC* and *virE*) enhance the process. Expression of the *vir* genes is induced by the low molecular weight phenolic compounds [e.g. acetosyringone (AS) or hydroxy-acetosyringone (OH-AS)] secreted from the cells of damaged plant tissues (Stachel et al., 1985).

The third component of the mechanism, the genes for bacterial attachment to the plant (*chvA* & *chvB*, *pscA*, *att*, and *cel*), is located in the host genome. Thus, the minimum requirements for *Agrobacterium*-mediated transfer of foreign genes into plants is the insertion of a foreign gene (replacing T-DNA) between RB and LB sequences of the T-DNA, combined with the production of trans-acting *vir* gene products and host gene products. These modifications of the Ti plasmid have been shown to be sufficient for the transfer of foreign DNA (Ream, 1989; Zambryski, 1992).

The second element of a plant transformation system is a mechanism for expressing foreign genes in plant cells. The mechanism in plants is analogous for the expression of foreign genes in animal, yeast, or bacterial cells. The foreign gene must be inserted between appropriate regulatory elements which will mediate the transcription of its DNA sequence into messenger RNA (mRNA), and translation of the mRNA into protein using the cell's own protein synthesis machinery. The specific regulatory elements differ between organisms. Promoters e.g., the 35S and 19S RNA promoters from the cauliflower mosaic virus (Paszkowski et al., 1984) and the mannopine synthase (*mas*) dual P1 and P2 promoters from TR-DNA (Velten et al., 1984), should be placed at the 5' end of the gene's coding sequence. The latter promoter system has the advantage that one promoter can be used to express the foreign gene and the other promoter used to express another gene. P1 and P2 promoters are induced by the plant hormone auxin or by wounding the plant tissue (Langridge et al., 1989 a). At the 3' end of the gene's coding sequence, polyA sequences should be added (1) for transcription termination and (2) for stabilization of the mRNA.

The third element of a plant transformation system is a mechanism for selecting and maintaining foreign genes in *E. coli*, *A. tumefaciens*, and in plant cells. Selectable markers, e.g., genes conferring resistance to a drug which normally kills the cell, are used for this purpose. The gene encoding neomycin phosphotransferase II, NPTII, (DeBlock et al., 1984;

Herrera-Estrella et al., 1983) selects for the presence of foreign genes in plants by conferring kanamycin resistance. The gene encoding β -lactamase, Ap^R, selects for the presence of foreign genes in *E. coli* by conferring ampicillin resistance and in *A. tumefaciens* by conferring carbenicillin resistance.

2.2 *Agrobacterium* binary plasmid-mediated gene transfer

All three elements of a plant transformation system could be incorporated in the Ti-plasmid; however, because of the very large size of the Ti-plasmid (approximately 200 kb), a binary plasmid system consisting of two different Ti-derived plasmids has been developed instead (Figure 1). The first plasmid, the Ti helper plasmid (about 180 kb) provides *vir* gene products *in trans* for the transfer of the second plasmid, the plant expression vector. The

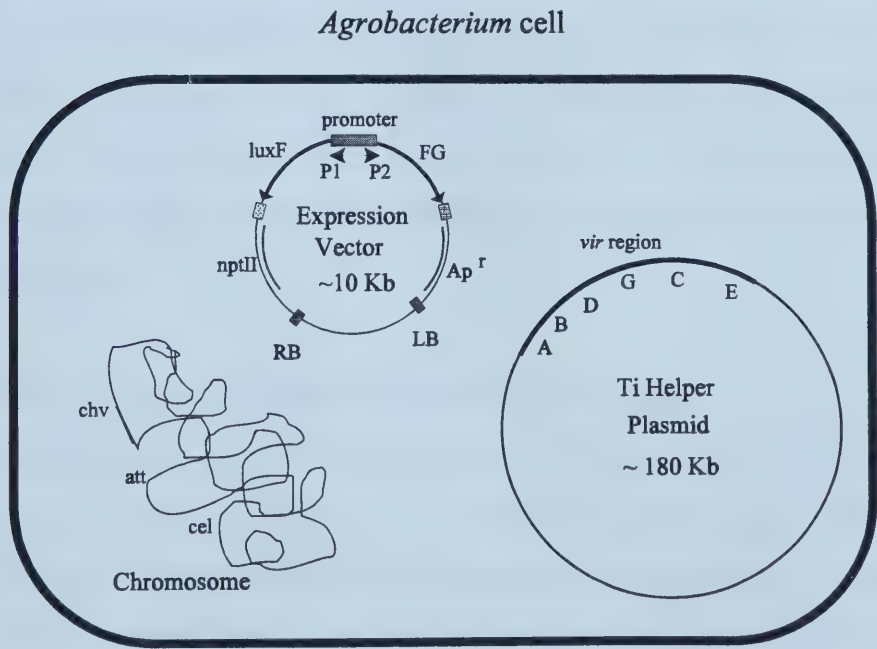


Figure 1. Binary plasmid system for *Agrobacterium*-mediated transformation of plants

entire T-DNA region including border sequences (about 20 kb) has been removed from the Ti helper plasmid so that plant tumor formation does not occur. The modified plasmid is used to replace the native Ti plasmid in the *Agrobacterium* strains selected as hosts for gene transfer to plants. The plant expression vector is amenable to genetic manipulations due to its smaller size (less than 10 kb). Only a small portion of the original Ti plasmid remains in this plasmid i.e., the right and left border sequences (25 bp each) to define the DNA segment to be transferred and the virulence and transfer origins. This plasmid also contains the necessary regulatory elements described above (e.g., P1 and P2, dual *mas* promoter; PA, polyadenylation sequences) for transcription of the foreign gene (FG) in plants and selectable markers for plasmid selection and maintenance in the different organisms (NPTII and Ap^R).

The overall gene transformation process requires several stages. First, the foreign gene is inserted into the plant expression vector DNA *in vitro*, then transferred into *E. coli* cells using bacterial transformation techniques. The plant expression vector is then transferred to *Agrobacterium* cells (containing the Ti helper plasmid) through conjugal transformation. Lastly, the transformed *Agrobacterium* cells — containing all essential components for foreign gene transfer — are incubated with wounded plant tissue to initiate plant transformation.

2.3 Broad host range for Agrobacterium-mediated gene transfer

The most important feature of the *Agrobacterium* binary plasmid system is its host range. The host range of *Agrobacterium* extends from most gymnosperms and dicotyledonous angiosperms to a number of monocotyledonous angiosperms (De Cleene and De Ley, 1976; De Cleene, 1985). *Agrobacterium*-mediated transformation has been most successful with tobacco, but works also with other plant species, such as petunia (Horsch et al., 1985), tomato (McCormick et al., 1986), potato (Shahin and Simpson, 1986),

Arabidopsis thaliana (Lloyd et al., 1986), and cotton (Umbeck et al., 1987). Recent work indicates *Agrobacterium* will even infect several species of graminaceous monocots, although at a reduced efficiency (Graves and Coldman, 1986). Thus, it appears most higher plants will be amenable to *Agrobacterium* binary plasmid-mediated genetic transformation.

2.4 Co-expression of foreign gene and lux reporter gene

Recent improvements in expression vector construction have included the co-expression of the foreign gene and of a "reporter" gene whose gene product can be readily detected and easily quantitated in cells. Two attractive reporter genes for plants include *E. coli* β -glucuronidase (GUS) gene and the luciferase genes, from fireflies (Ow et al., 1986) or *Vibrio harveyi* (Koncz et al., 1987). Expression of GUS can be accurately measured in fluorometric assay on a very small amount of transformed plant tissue. Expression of luciferase has the further advantage of being detected *in vivo* without disturbing the development of the plants using low light image microscopy (Langridge et al., 1989b).

The luciferase genes involved in bioluminescence have been isolated and expressed in *E. coli*. The best characterized genes are the related *luxA* and *luxB* genes from *Vibrio harveyi*. The *V. harveyi* luciferase [alkanal monooxygenase; alkanal, reduced-FMN:oxygen oxidoreductase (1-hydroxylating, luminescing), EC 1.14.14.3] is a heterodimer, composed of α (*luxA*) and β (*luxB*) polypeptide subunits (Hastings et al., 1977), which catalyzes the oxidation of long-chain fatty aldehydes. The reaction requires reduced flavin mononucleotide (FMN) and molecular oxygen and results in the emission of blue-green (490 nm) light (Ziegler et al., 1981). The overall reaction is:



Because the long-chain aldehyde substrate can readily enter living cells, this reporter

enzyme allows *in vivo* analysis of gene expression. This is particularly important when studying gene regulation in multicellular eukaryotes such as plants. By expressing the *luxA* and *luxB* cistrons from a divergently transcribed dual promoter such as the P1 and P2 promoters of mannopine synthetase (*mas*), the *V. harveyi* luciferase gene has been shown to produce luciferase-mediated light emission in protoplasts, in calli, and in the leaves of transformed plants (Koncz et al., 1987; Koncz et al., 1990). The necessity of expressing two *lux* genes from independent promoters has been overcome by successfully fusing the *luxA* and *luxB* genes into one translational unit encoding a single fusion protein with both activities (Olsson et al., 1989). One of the *luxAB* fusion constructs (*lux F*) has been reported to be active in *E. coli* (Escher et al., 1989); *Bacillus megaterium* and *Bacillus thuringiensis* (Wang, et al., 1993), and in the tobacco plant *Nicotiana tabacum* (Jiang et al., 1992).

The mannopine synthase (*mas*) promoter is a dual promoter from T-DNA functions in transformed plant tissues (Velten et al., 1984). The expression of luciferase genes under the control of *mas* promoters P1 and P2 was enhanced by wounding and was differentially affected by auxins and cytokinins in normal and tumorous plant tissues (Langridge et al., 1989).

The *luxF* gene fusion under the control of one *mas* promoter and a foreign gene of interest under the control of the other *mas* promoter now permits the simultaneous induction and expression of both luciferase activity and the foreign gene product in the same plant cell. From measurements of luciferase activity on living plant tissues at different stages of plant development, investigators can now infer (indirectly) the location of the foreign gene in specific tissues and its inducibility throughout plant development. Furthermore, since the relative strengths of the P1 and P2 *mas* promoters are known, a preliminary guesstimate can be made of the amount of foreign gene expression which may be occurring in the same cells. Molecular genetic techniques, which permit the specific detection of foreign gene DNA

sequences, messenger RNA transcripts, and protein can be then be used to confirm and quantitate foreign gene expression in these tissues.

3. Early Studies With Transgenic Plants

3.1 *Potential benefits of transgenic plants*

These and other plant transformation techniques are being applied to solve problems such as insect resistance and tolerance to cold. Insect damage, drought, and unexpected climatic changes can result in damaged crops, economic losses, and in the worst case, famine. The continued use of pesticides is costly, and in general, the potential damage to the environment. Increased tolerance to adverse environmental conditions e.g., cold tolerance, might permit food crops to be grown in a broader geographic area and/or for longer growing seasons.

3.2 *Introduction of genes for *B. thuringiensis* insect toxins into plants*

The *cry* genes of *Bacillus thuringiensis* were an attractive choice for plant biotechnologists engineering the first transgenic plants. The parasporal crystal (Cry) proteins formed during sporulation in *B. thuringiensis* are natural bioinsecticides with no harmful effects on humans, plants, animals, pollinators, or predators (Cakmakci, 1986). They are highly selective for certain insects e.g., the *CryI* is active against Lepidoptera, *CryII* is active against Lepidoptera and Diptera, *Cry III* is active against Coleoptera, and *Cry IV* is active against Diptera (Hofte and Whiteley, 1989). Thus far, this bioengineering approach to insect control appears successful; however, it has required modifications of the *cry* genes and numerous other refinements in the initial expression strategy (Sutton et al., 1992). The insecticidal activity of these recombinant bacterial toxins has been assessed in tomato (Delannay et al., 1989), potato (Perlak et al., 1993), cotton (Wilson et al., 1992; Benedict,

1993), corn and tobacco (Warren et al., 1992; Carozzi et al., 1992) plants. The insect resistance of these transgenic plants demonstrates that a plant can benefit from expression of a bacterial protein.

3.3 Introduction of gene for fish antifreeze protein into plants

The production of cold tolerant plants has been even more challenging. The identification of natural antifreeze proteins in cold water fish led to the cloning of the antifreeze genes (Davies and Hew, 1980; Lin and Gross, 1981; Davies et al., 1982). The creation of transgenic plants expressing antifreeze protein is the first approach that has been taken to bioengineer plants which are more resistant to cold. A synthetic flounder antifreeze gene was designed and fused to the chloramphenicol transferase (CAT) gene under the control of the cauliflower mosaic virus (CaMV) 35S promoter and introduced into corn protoplasts (Georges et al., 1990). Antifreeze messenger RNA was present in transgenic tobacco but no antifreeze protein was detected and no inhibition of ice recrystallization was evident in the leaves (Hightower et al., 1991). Subsequent experiments with transgenic plants extracts demonstrated that production of antifreeze protein is cold-dependent, possibly due to requirements for a specific secondary structure (Kenward et al., 1993). While the antifreeze protein is promising, it is not necessarily the only approach to increasing cold tolerance in plants.

4. Bioengineering Cell Membranes for Cold Tolerance

Plants are sensitive to changes in ambient temperature because they are poikilotherms and sessile, thus they cannot escape the stressful temperatures. Temperature stresses are divided to "chilling" and "freezing" stresses. Chilling stress is caused by temperature from 0°C to 15°C. Plants can be injured or killed at such temperatures. Freezing stress is caused

by subzero temperatures that result in severe injuries either in the absence or the presence of ice (Levitt, 1980c).

Chilling temperatures are known to cause injuries include membrane damages, such as the lateral phase separation of non-fluid membrane components (Lyons et al., 1979), photoinhibition (Hurry et al., 1993), protein/enzyme denaturations (Guy, 1990), and accumulation of toxic compounds, such as free radicals (Monk et al., 1989). Injuries caused by freezing temperatures mainly consist of membrane damages (Steponkus, 1990), inactivation of membrane pumps (Palta and Weiss, 1993), and cell wall-plasma membrane separations (Johnson-Flanagan and Singh, 1986).

4.1 Cell membrane fluidity is lost at low temperature

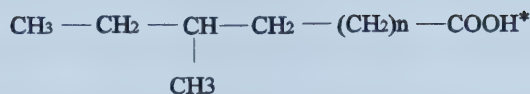
Physiological manifestations of environmental stress in plants often implicate cell membranes as the primary site of injury (Levitt, 1980a,b). At low temperatures, there is a decrease in membrane fluidity (Overath et al., 1976; Raison and Orr, 1986) which leads to a decrease in the activity of membrane-bound enzymes (Raison et al., 1971; McMurchie and Raison, 1979) and loss of semipermeable membrane properties (Haest et al., 1972; Blok et al., 1975). A phase transition in membrane lipids from a fluid, or liquid-crystalline state, to a more solid phase has been offered as the explanation for impairment of membrane functions (Mateu et al., 1978). Membrane lipids satisfy the need for membrane fluidity, structure, and permeability, as well as protein association and function. The most abundant lipid in plasma membranes is phospholipid, even the composition of phospholipids vary in other organelle membranes. The amphipathic (or amphiphilic) nature of phospholipids — possessing both a hydrophilic (polar) end and a hydrophobic (nonpolar) end — is essential to the formation of the lipid bilayer.

4.2 Fatty acid composition determines membrane fluidity

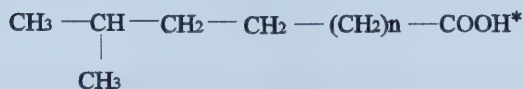
The nature of the acyl chain region comprising the hydrophobic domain of most membrane lipids determines membrane fluidity. This domain contains fatty acids, either belonging to the straight-chain or branched-chain families (Figure 2). The straight-chain fatty acid family includes saturated fatty acids consisting of 16-20 carbons (C_{16} - C_{20}) and unsaturated fatty acids that contain one or more double bonds. Common straight-chain fatty acids are palmitic ($C_{16:0}$), stearic ($C_{18:0}$), oleic ($C_{18:1}$), linoleic ($C_{18:2}$), and linolenic ($C_{18:3}$) acids. The branched-chain fatty acid family contains iso-, anteiso- and ω -alicyclic fatty acids with or without a substitution (unsaturation and hydroxylation). Terminal methyl-branched fatty acids, 13-methyl-tetradecanoic (iso- C_{15}) and 15-methylhexadecanoic (iso- C_{17}) in *Bacillus subtilis* (natto) (Saito, 1960) and 12-methyltetradecanoic (anteiso- C_{15}) acid in *Sarcina* (Akashi and Saito, 1960), were first identified in 1960. Several years later, three additional branched-chain fatty acids, 12-methyltridecanoic (iso- C_{14}), 14-methylhexadecanoic (anteiso- C_{17}), 14-methylpentadecanoic (iso- C_{16}), were discovered in *B. subtilis* (Kaneda, 1963).

A clear difference between straight-chain and branched-chain fatty acid containing cell membranes is the mechanism that controls their fluidity. The fluidity of membranes composed of straight-chain fatty acids is adjusted to the proper level by the unsaturated fatty acids which is normally located on the 2-position of the phospholipid molecules, whereas the fluidity of membranes with branched-chain fatty acids is controlled mainly by 12- and 13-methyltetradecanoic acids. Thus, bacteria with the straight-chain membrane system usually requires unsaturated fatty acids for growth, but these unsaturated fatty acids are not essential for bacteria with the branched-chain membrane system. Many bacteria, such as the very successful *Bacillus* genus, the majority of fatty acids imparting membrane fluidity are branched-chain fatty acids, instead of unsaturated fatty acids. These branched-chain fatty

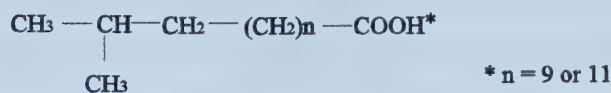
Branched-chain anteiso series



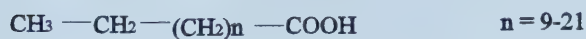
Branched-chain odd numbered iso series:



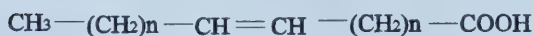
Branched-chain even numbered iso series:



Straight-chain saturated fatty acids:



Straight-chain monounsaturated fatty acids: $n = 5-7$



Straight-chain polyunsaturated fatty acids:

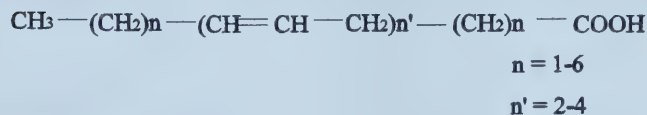


Figure 2. Branched-chain fatty acids and straight-chain fatty acids

acids have physical properties similar to those of unsaturated fatty acids. *E. coli* mutants which cannot synthesize unsaturated fatty acids lose membrane fluidity and cannot grow unless supplemented with unsaturated fatty acids in their growth medium. These bacterial mutants have been reported to grow if supplemented with branched-chain fatty acids instead of straight-chain unsaturated fatty acids (Kaneda, 1991; Jackowski et al., 1991).

4.3 Branched-chain fatty acids have lower transition temperatures

The fluidity of membrane lipids can be described by the phase transition temperature (T_m). Phase transition temperature (T_m) has been measured for lipids containing different types of fatty acids (Kaneda, 1977). It was found that the phase transition temperatures of saturated straight chain fatty acids and the branched-chain iso-fatty acids with the same number of carbons were significantly different (Table 1). Iso-acyl phosphatidylcholine has a T_m which ranged from 18-28°C below that of the corresponding normal saturated acyl phosphatidylcholine (Silvius, 1982). Thus, differences in lipid content of saturated versus branched-chain fatty acids would be expected to make a difference in the fluidity of cell membranes.

Table 1. Phase Transition Temperature of Diacylphosphatidylcholine

	Fatty Acid	Diacylphosphatidylcholine T_m (°C)
Straight-Chain	n-C ₁₄	24.0
	n-C ₁₅	34.2
	n-C ₁₆	41.5
	n-C ₁₇	48.8
	n-C ₁₈	54.8
Branched-Chain	Iso-C ₁₄	6.5
	Iso-C ₁₅	6.5
	Iso-C ₁₆	22.0
	Iso-C ₁₇	27.0
	Iso-C ₁₈	36.5
	Anteiso-C ₁₅	-16.5
	Anteiso-C ₁₇	7.6
	ω-Cyclohexyl-C ₁₇	18.3
	ω-Cyclohexyl-C ₁₉	34.9

Studies of cold tolerant microorganisms suggest that fatty acid composition of their cell membranes differs from that of their counterparts adapted to warmer ecological niches. Membrane fatty acid analysis of Antarctic bacteria showed that the branched-chain fatty acids are predominant in the membrane fatty acids in the psychrophiles (Rotert et al., 1993). Deep-sea barotolerant bacterium contains branched-chain fatty acids as a major component of membrane lipids (Kaminura et al., 1993). *Legionella shakespearei* sp., isolated from cooling tower water contained cellular branched-chain fatty acids as the major lipid components (Verma et al., 1992). The proportion of branched-chain to saturated straight-chain acids and those of anteiso- to iso- branched-chain acids is increased with reducing temperature instead of fatty acid unsaturation. These findings suggest that membrane fluidity could be improved at low temperature by increasing the content of branched-chain fatty acids in cell membranes.

4.4 Differential distribution of branched-chain fatty acids in cell membranes

Although both straight-chain and branched-chain fatty acids are synthesized by almost all organisms, they are differentially distributed in cell membranes. Branched-chain fatty acids are normally found only in the cell membranes of some bacteria, a few protozoa, and a few fungi; straight-chain fatty acids are found in the cell membranes of plants, animals, and most bacteria and fungi (Brennan, 1988; Kaneda, 1977; Lechevalier, 1977; O'Leary and Wilkinson, 1988; Wilkinson, 1988).

The study of branched-chain fatty acids has focused primarily upon the genus *Bacillus* due to their high content of these fatty acids (Table 2). All 22 out of 48 species of *Bacillus* examined by Kaneda (1977) contained 60-90% iso- and anteiso- saturated fatty acids as major acid components of lipids. More recently, Kaneda (1991) has reported that 56 out of 398 genera in *Bergey's Manual of Systematic Bacteriology* possess branched-chain

fatty acids in excess of 20% of total cellular fatty acids.

Table 2. Percentage of branched-chain fatty acids in microorganisms

Gram-positive	<i>Bacillus</i> : <i>B. subtilis</i> (95%) <i>B. natto</i> (88%) <i>B. thuringiensis</i> (81%) <i>B. megaterium</i> (89%) <i>B. lichniformis</i> (91%) <i>B. polymyxa</i> (70%) <i>Corynebacterium manihot</i> (93%) <i>Listeric grayi</i> (65%) <i>Arthrobacter oxydans</i> (74%)
Gram-negative	<i>Bacterioide asaccharolyticus</i> (96%) <i>Legionella jordanis</i> (84%) <i>Pseudomonas putrefaciens</i> (48%) <i>Spirochaeta litoralis</i> (68%) <i>Flavobacterium thermophilum</i> (99%)
Prosthecate gliding bacteria	<i>Cytophaga aquatilis</i> (49%) <i>Myxococcus stipitatus</i> (68%)
Actinomycetes	<i>Micromonospora chalcea</i> (88%) <i>Streptomyces gelaticus</i> (79%) <i>Thermomonospora curvata</i> (72%)

In plants, long branched-chain fatty acids (e.g. C₂₅-C₃₅) are found primarily in the cuticle waxes of plant leaves and stems but not in cell membranes (Kaneda, 1968). Radunz (1986), however, has reported finding branched-chain iso- and anteiso-fatty acids in the membranes of yellow-white leaves and petals of the plastome mutants "*Prasinizans*" of *Antirrhinum majus* and "*Xanthi*" of *Nicotiana tabacum*. Thus, branched-chain fatty acids

which are not normally found in the green tissues of the wild-type plants are able to locate these tissues in these mutants. These branched-chain fatty acids have taken the place of some of the straight-chain unsaturated fatty acids which would normally be there and further increase cell membrane fluidity.

4.5 Plants and bacteria have the same type of fatty acid synthetases

Fatty acids are synthesized by a series of enzymatic reactions that are catalyzed by fatty acid synthetases. Fatty acid synthetases from a wide variety of sources are divided into two groups on the basis of their physical structures. Type I group includes the synthetases with a multi-functional enzyme complex (Figure 3). The fatty acid synthetase system from animals, yeasts and fungi belong to this type of system. Type II group, also called the soluble system, is composed of seven individual enzymes (Table 3). Type II includes fatty acid synthetases from higher plants and bacteria. Branched-chain fatty acid synthetases also belong to this group (Kaneda, 1991).

Table 3. Enzymes in Type II Fatty Acid Synthetase System

ENZYME
1. Acetyl-CoA Carboxylase
2. Malonyl-CoA:ACP Transacylase
3. Acetyl-CoA:ACP Transacylase
4. β -keto acyl-ACP synthase
5. β -keto acyl-ACP Reductase
6. β -hydroxyacyl-ACP dehydrase
7. Enoyl-ACP Reductase

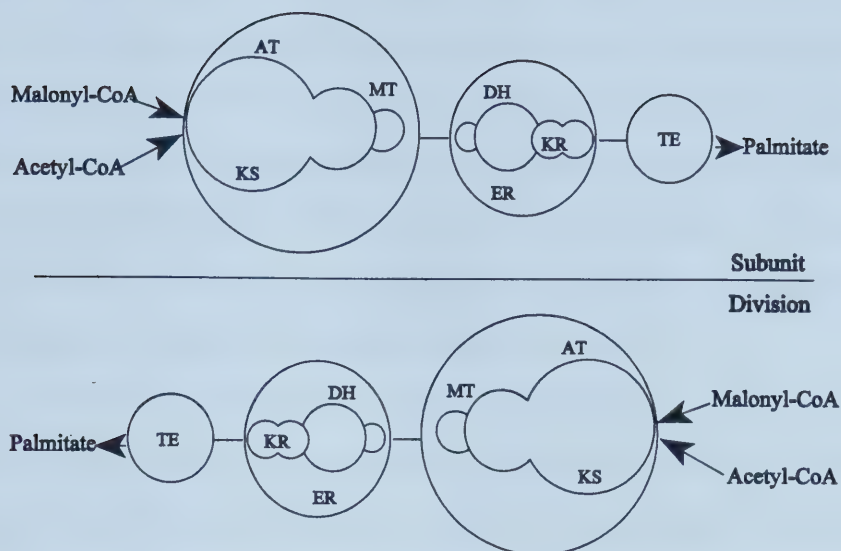


Figure 3. Proposed functional map of the chicken fatty acid synthetase type I (Vance & Vance, 1991). The abbreviations for partial activities are: AT, acetyl transferase; MT, malonyl transferase; KS, β -ketoacyl synthase; KR, β -ketoacyl reductase; DH, dehydrase; ER, enoyl reductase; TE, thioesterase; ACP, acyl carrier protein; Mal, malonyl; Ac, acetyl.

4.6 Comparison of straight-chain and branched-chain fatty acid biosynthesis

Biosynthesis of straight-chain fatty acids has been extensively studied in a wide variety of organisms (Bloch & Vance, 1977; Volpe & Vagelos, 1976). The overall synthetic pathway is strikingly uniform throughout animals, plants, and microorganisms. In the synthetic reactions, acetyl-CoA is used as the primer and its carbon chain is elongated by the repeated condensation of malonyl-CoA to the primer, yielding palmitic acid as the major product. In bacteria, synthesis is carried out in the cytosol, whereas in higher plants, fatty acid synthetases are located in leaf tissues of chloroplasts and in proplastids, in seeds and mesocarp tissues although the enzymes are nuclear encoded.

Studies of the biosynthesis of branched-chain fatty acids in bacteria were initiated at about the same time as those of palmitic acid synthesis, but the full understanding of branched-chain fatty acid biosynthesis remains to be established. The slow progress is mainly due to the low activity of the enzyme systems in *Bacillus*. It has been reported that the overall rate of branched-chain fatty acid synthesis in *B. subtilis* is only 1/50 of the combined rate of straight-chain saturated and monounsaturated fatty acid synthesis in *E. coli* (Kaneda, 1991).

Synthesis *de novo* of branched-chain fatty acids still remains to be established. It has been proved that branched-chain fatty acids in bacteria are synthesized by a mechanism similar to that of straight-chain fatty acid synthesis in *E. coli*. The only difference between the systems appears to be the substrate specificity of acyl-CoA:ACP transacylase. Once an ACP derivative of acyl intermediates is formed, its elongation reaction can be carried out by a system identical to that of *E. coli* (Kaneda, 1991).

There are three types of primer sources involved in synthesis of branched-chain fatty acids in bacteria. The first type includes branched-chain α -oxo acids, such as valine, leucine and isoleucine. They are used by nearly all bacteria with branched-chain fatty acids as lipid

components. The second type includes branched short-chain carboxylic acids used by a small number of bacteria that are incapable of utilizing branched-chain α -oxo acids as primer sources. These branched short-chain carboxylic acids must be exogenously supplied for the proper growth of such bacteria. The third type of primer source is composed of species of bacteria whose cellular fatty acids are mostly ω -alicyclic fatty acids. In this case, cyclic carboxylic acids are used to synthesize alicyclic fatty acids by a mechanism similar to that used by branched short-chain carboxylic acid as primer sources (Krischke, 1990). Among the three, the first type of α -oxo acids as primer sources are the most extensively studied.

The terminally branched-chain acids are made by substituting isobutyryl-CoA or 2-methylvaleryl-CoA for acetyl-CoA as the primer of fatty acid biosynthesis. The branched-chain acyl-CoA primer is transacylated to ACP and is used as the acceptor of malonyl groups in elongation cycles of fatty acid synthesis. The methyl branches which originate in the primer molecule used by the fatty acid synthetase (FAS) enzyme system, are usually derived from an amino acid. For instance, leucine gives rise to an iso-branched fatty acid and isoleucine to an anteiso-branched fatty acid. These three amino acids are first converted to keto acids by transaminases that are probably specific for the amino acid residue. Then the enzyme, branched-chain α -oxo acid dehydrogenase (BCOADh), converts the keto acids to their respective CoA thioesters. These are in turn converted to acyl carrier protein (ACP) thioesters by the acyl-CoA:ACP transacylase prior to acceptance by the FAS enzyme system (Boudreaux et al., 1981). The elongation of branched-chain fatty acid precursors takes place by the same sequence of reactions established for the synthesis of straight-chain fatty acids by in the *E. coli* system (Kaneda, 1977).

The proposed pathway for the synthesis of branched-chain fatty acids in *B. subtilis* (Kaneda, 1991) uses many of the same enzyme reactions as the pathway for the synthesis of straight-chain fatty acids (Figure 4). The straight-chain fatty acid synthesis pathway begins

in column A. The branched-chain fatty acid synthesis pathway begins with branched-chain α -oxo acids in column B. Both pathways then require the same reactions shown in the central column. Reactions 1-7 are catalyzed by the seven soluble enzymes of type II synthetase. Reaction eight is catalyzed by branched-chain α -oxo acid decarboxylase (BCOADc). Reaction nine remains to be elucidated. It is assumed that the primer is an aldehyde thiamine pyrophosphate derivative or an aldehyde-enzyme complex, which condenses with a malonyl-ACP derivative. Once the β -hydroxyacyl-ACP derivative is formed, the remaining reactions are carried out by the same processes as in the straight-chain system (Kaneda, 1991).

4.7 BCOADc catalyzes a critical step in branched-chain fatty acid synthesis

Initial studies on synthesis of branched-chain fatty acids from α -oxo acids (also called α -keto acids) as primer sources suggested that cofactors CoA and NAD are not required for the synthesis of fatty acids from α -oxo acid substrates; instead they inhibit the synthesis (Kaneda, 1973). Thus, α -oxo acid substrates (e.g. valine, leucine, and isoleucine) are decarboxylated, but not dehydrogenated, to yield the actual primers for fatty acid synthesis. In support of this mechanism, two α -oxo acid decarboxylases (BCOADc and pyruvate decarboxylase) were isolated from cell extracts of *B. subtilis*. Immune-precipitation experiments demonstrated that BCOADc, not the pyruvate decarboxylase, is essential for the incorporation of branched-chain α -oxo acid substrates into fatty acids (Oku & Kaneda, 1988). Meanwhile, the synthesis of acyl-CoA esters from branched-chain α -oxo acids by BCOADc (present in *B. subtilis*) requires CoA and NAD as cofactors (Namba et al., 1969). These results suggest that the decarboxylase essential to fatty acid synthesis from α -oxo acids (BCOADc) may be a free form of dehydrogenase (E1) of the branched-chain α -oxo acid dehydrogenase (BCOADh) complex. BCOADc is a heterodimer of subunits: two α

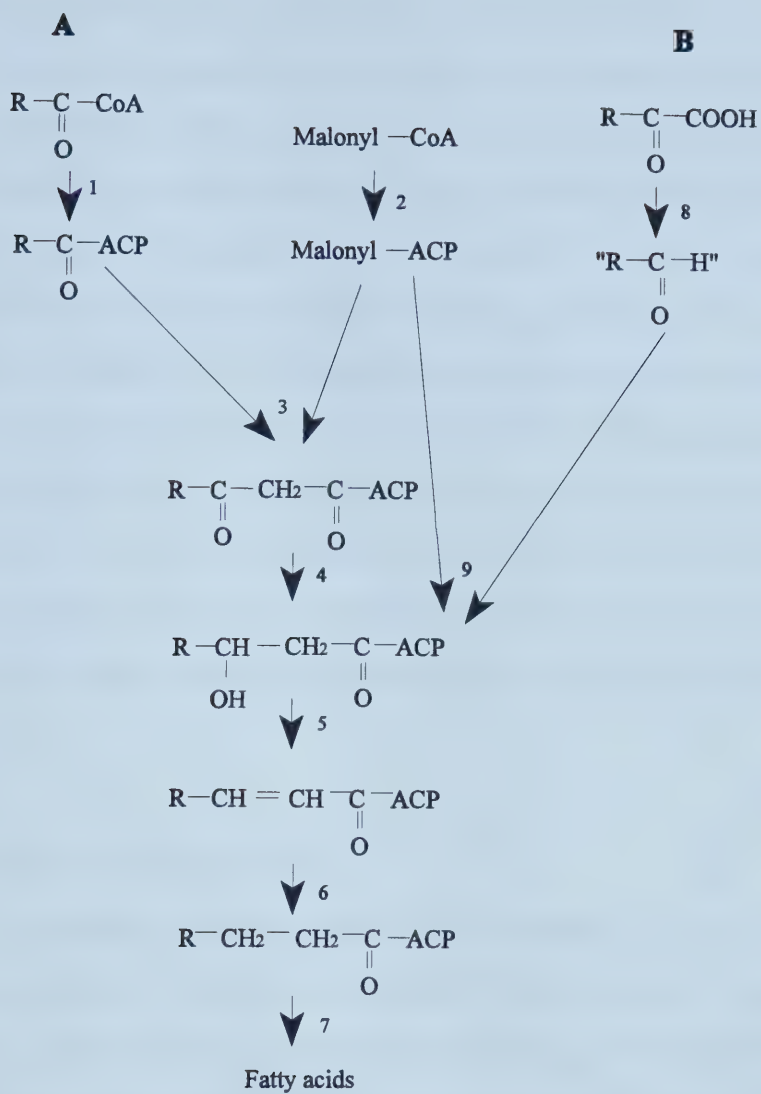


Figure 4. Comparison of straight-chain and branched-chain fatty acid synthesis pathways. Straight-chain pathway (column A + central column); branched-chain pathway (column B + central column).

subunits (E1 α) and two β subunits (E1 β), with a molecular weight of 144.4 KDa.

To pursue this hypothesis further, a project was undertaken with the *B. subtilis* mutant strain CAC-11. This bacterium is unable to grow without supplementation with branched short-chain carboxylic acids because it lacks the activity of branched-chain α -oxo acid dehydrogenase (Willecke and Pardee, 1977). CAC-11 was used to identify and clone the genes encoding branched-chain α -oxo acid dehydrogenase in *Bacillus subtilis* (Wang et al., 1993). The cloning and DNA sequencing were carried out mainly in the laboratory of Dr. T. Kaneda at the Alberta Research Council (Edmonton, Alberta, Canada). These genes were completely sequenced and the data analyzed after I joined the graduate program in the laboratory of Dr. A.A. Szalay, Department of Plant Science at the University of Alberta on September 1, 1991. For the sake of completeness and clarity, strategies used for both the cloning and sequencing of the BCOADh genes (and subset of BCOADc genes) are enclosed in this thesis.

5. Project Goal and Objectives

The goal of this project was to increase plant tolerance to cold temperature. Based on intrinsic properties of cell membrane lipids and comparison of their fatty acid composition, an hypothesis was formed that the introduction of branched-chain fatty acids into the lipids of plant cell membranes would improve membrane fluidity, and thus cell survival, at lower temperatures. The strategy for testing this hypothesis was to augment the fatty acid biosynthetic capacity of tomato plant cells with foreign genes which might provide a key step for branched-chain fatty acid synthesis into membrane lipids. This hypothesis was testable only if such foreign gene(s) could be identified, cloned, and transferred to plants.

The major objectives of this project were (1) to clone and sequence the BCOADc genes of the BCOADh complex from *B. subtilis*; (2) to generate transgenic tobacco and

tomato and plants from *Agrobacterium*-mediated binary plasmid transformation using the BCOADc genes and the *lux* (luciferase) marker genes; (3) to confirm gene transfer and function using the Southern genomic DNA hybridization analysis and luciferase assay; (4) to carry out Northern hybridization analysis of BCOADc gene and *lux* gene transcription levels in transgenic tomato plants; (5) to determine the location of luciferase protein in leaf, flower, and fruit tissues of transgenic tomato plants by luminometry and low light image analysis; (6) to detect BCOADc and luciferase gene products by Western (immuno) blot analysis; and (7) to evaluate the condition of luciferase activity in transgenic tomato plants grown at 4°C compared to 22°C.

MATERIALS

1. Bacterial Strains

1.1 *Escherichia coli*

E. coli LE392 [*supE44 supF58 hsdR514 galK2 galT22 metB1 trpR55 lacY1*] was used to propagate bacteriophage vectors and their recombinants (Borck et al., 1976; Marie et al., 1977). JM103, a restriction-deficient derivative of JM101, was used to propagate bacteriophage M13 recombinants (Messing et al., 1981). MV1193 [(*lac-proAB*) *rpsL thi endA spcB15 hsdR4 (srl-recA) 306 :: Tn10 (tet^r)*] was used to obtain single-stranded copies of phagemids pUC118 and pUC119 (Zoller, et al., 1987). Plasmid production was carried out in DH5 α [*supE44 Δ lacU169(Φ 80 Δ lacZ M15) hsdR17 recA1 endA1 gyrA96 thi-relA1*] (Hanahan, et al., 1983). S17-1 [RP 4-2 (Tc::Mu) (Km::T7) Tp Sm Pro *res- mod⁺ recA*], which modifies plasmid DNA, was used as the donor for the transfer of plasmids to *Agrobacterium* through conjugal transformation. DLT101 (LE392 Δ uncBD::Tn10 *recA56 F⁻ hsdR gal⁻ Sn1::Tn10 rec56 ∇ lD69 lacUV5-T7 gene 1*) strain was used for production of α and β subunits of BCOADc (branched-chain α -oxo acid decarboxylase). It is a *recA* derivative of BL21(DE3) which overproduces T7 RNA polymerase following induction with IPTG (Studier et al., 1986).

1.2 *Agrobacterium tumefaciens*

GV3101 (pMP90RK) is a Rif^r recipient strain containing plasmid pMP90 (Gm^r). In this deletion derivative of Ti-plasmid pTiC58, the T-DNA genes have been replaced by plasmid pBR322 sequences and RK-2 genes. The PK-2 gene from plasmid pRK2013 (Km^r) encodes helper (*tra*) functions permitting conjugal transfer of plasmid DNA from *E. coli* to *Agrobacterium*.

2. Bacteriophages

Helper phage M13K07 was used to infect *E. coli* MV1193 for the production of single-stranded phagemid DNA used in DNA sequencing. Bacteriophage λ was used to construct a genomic library of *B. subtilis* 168s and for cloning BCOADh genes.

3. Plant materials

Nicotiana tabacum Petit Havana SR1 (Maliga et al., 1973) is a common cultivar for generation of transgenic plants. It has been used as a recipient plant for transformation of foreign genes, such as the luciferase gene and BCOADc gene cassettes. *Lycopersicon esculentum* var. Roma, a tomato plant with egg-shaped fruit, was used for transforming plants with the bacterial luciferase fusion gene and the BCOADc gene cassettes.

4. Plasmids

4.1 Cloning and DNA sequencing of BCOADh and BCOADc genes

Plasmids pUC118 and pUC119 were used for cloning and DNA sequencing the BCOADh genes. pBluescript II KS +/- (Stratagene), an *E. coli* cloning vector, was used mainly for subcloning and DNA sequencing of the A and B gene fragments of BCOADc.

4.2 Transformation of the BCOADc genes into plants

Plasmid pPCV701 (Figure 5), a plant expression vector containing mannopine synthase gene promoters (dual *mas* promoter, Koncz, et al., 1987), was used to drive the BCOADc A and B genes and to generate the pPCV701-AB transformed tomato and tobacco plants. Plasmid pPCV701-*luxFP1*, a pPCV701 derivative containing the bacterial luciferase fusion gene *luxF* driven by P1 promoter of *mas* dual promoter system (Jiang, et. al., 1993), was used to generate pPCV701-*luxFP1*-B transformed tobacco and tomato plants.

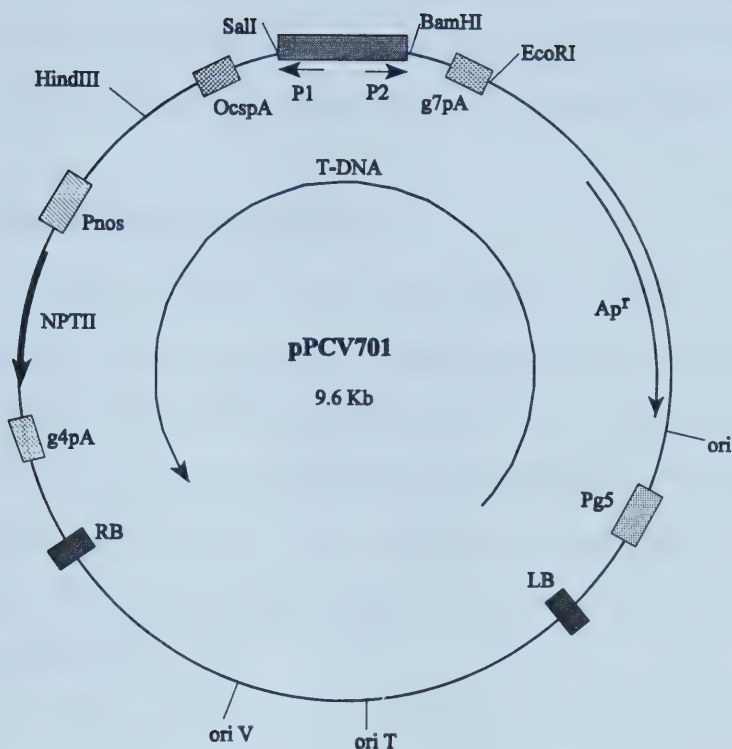


Figure 5. Circular map of plant expression vector pPCV701. P1 and P2 indicate the dual promoters of *mas* promoter cassette; OcsA, g7pA, g4pA and pg5 are the polyA sequences used to terminate genes driven by the P1 and P2 promoters, Pnos promoter and ampicillin resistant gene (Ap^r) cassette; NPTII is neomycin phosphotransferase gene II presenting kanamycin resistance phenotype; RB and LB are the 25 bp right border and left border sequences isolated from T-DNA of *Agrobacterium*; the T-DNA is indicated as an opened circle between the RB and LB sequences; ori is a bacterial origin; oriV and oriT are virulence and transfer origins, respectively; SalI, BamHI, HindIII and EcoRI are the important cleavage sites of restriction enzymes used in this study.

4.3 Production of E1 α and E1 β subunits of BCOADc in vitro

Plasmids pBluescript II KS +/- was used for overproduction of α and β subunits of BCOADc proteins in *E. coli* DLT101. This vector was designed for T7 RNA polymerase transcription of a DNA fragment under the control of T7 promoter (Studier, et al., 1990).

5. *Bacillus subtilis* genomic library

The genomic DNA library used in these studies was constructed from *Bacillus subtilis* 168s (*trpA hisH smo-1 tet-1*) by T. Kuriki in the laboratory of T. Kaneda, Alberta Research Council. A 920-bp PstI genomic fragment from *B. subtilis* 168s, subcloned in shuttle vector pAC32R2, was used as a probe for cloning the BCOADh (branched-chain α -oxo acid dehydrogenase) genes. D. Trivers, also in the laboratory of T. Kaneda, has demonstrated that this fragment complements the *bfmB* mutation in *Bacillus subtilis* CAC-11 (*strA hisH bfmB smo-1*).

6. Oligonucleotide Primers

6.1 Primers for sequencing of BCOADh genes

- #1: 5'-AATGGAACGAGATTCTC-3'
- #2: 5'-ATTGTCATGCCATCAAC-3'
- #3: 5'-CATCCAGCATGGTTTGTT-3'
- #4: 5'-TTAACCGCTTGATAAA-3'
- #5: 5'-GCAATCAATTTGGCGATG-3'
- #6: 5'-AGGCTGCGATATCCTT-3'
- #7: 5'-GACATTACTGACATTTG-3'
- #8: 5'-GCTTCGACTGATTGAGA-3'
- #9: 5'-TGGCTTCTTTATCAAGC-3'
- #10: 5'-ACAAGCCATTTGCTGAT-3'
- #11: 5'-CAAACGGAGAACAGCT-3'
- #12: 5'-GAACGAACCTGTGTTG-3'
- #13: 5'-CGCTGAATAATAAAGCAT-3'
- #14: 5'-TACGGCAATACATAATCC-3'
- #15: 5'-CTGGACGCAAACACCGT-3'

#16: 5'-GTTAAACCGTTCTGGCA-3'
 #17: 5'-GGTTACAACGCAAGTGC-3'
 #18: 5'-TAAGCAAGTCGCATGTG-3'
 #19: 5'-TGCTTGATGTCGGTTTG-3'
 #20: 5'-GAAAATACGGATCGCTG-3'
 #21: 5'-GTTCATAGAGTCCCGCT-3'
 #22: 5'-ACCTGCAAGCCGTTTGA-3'
 #KLR62: 5'-CCGGCTATGCCTTATG-3'
 Uni. Primer: 5'-GTTTTCCAGTCACGAC-3'
 Rev. Primer: 5'-AACAGCTATGACCATG-3'

6.2 Primers for subcloning of the DNA fragments of BCOADc genes

A1: 5'-CGGTCGACATGAGTACAAACCGACATCA-3'
 A2: 5'-CGGTCGACCTACTTCGCATAAACATAAT-3'
 B1: 5'-CGGGATCCATGTCAGTAATGTCATATAT-3'
 B2: 5'-CGGGATCCTTAAAACTCCGCTAATTCTC-3'
 ASD: 5'-CGGTCGACAAGGAGGGGCTTGAATGAGTA-3'
 BSD: 5'-CGGGATCCAGGGAGGAAGAACAAATGTC-3'

6.3 Primers for detection of mRNA of BCOADc genes and luxF gene in plants

Primer #14 was used for detection of messenger RNA from A gene of BCOADc enzyme. Primer #21 was used for detection of mRNA from B gene of BCOADc enzyme. Primer "luxA1 bottom" (5'-CCTTCAGACGCTTTGCCCAG-3') was used for detection of luxF mRNA in plants; Primer "U6- tomato" (5'-TTGGACCATTCTCGATTTG-3') was used as a positive control primer to detect the quality of total RNA and the reaction systems during experiments.

7. Reagents and Enzymes

7.1 Antibiotics and plant hormones

Table 4. Antibiotics

NAME	ABR.	CONCENT. IN MEDIUM ($\mu\text{g/ml}$)	STOCKS (mg/ml)	SOLVENT
Ampicillin	Ap	100	50	H ₂ O
Carbenicillin	Cb	100	50	H ₂ O
Tetracycline	Tc	25	1.5	50% EtOH
Kanamycin	Km	25 (<i>Bacillus</i>) 25 (<i>Agrobacterium</i>) 100 (Plants)	50	H ₂ O
Gentamicin	Gm	25	10	H ₂ O
Rifampicin	Rif	100	50	EtOH
Streptomycin	Sm	150 (<i>Pseudomonas</i>)	100	H ₂ O
Nalidixic acid		15 (<i>Pseudomonas</i>)	15	H ₂ O
Claforan	Cla	300	-	-

Table 5. Plant Hormones

NAME	ABR.	CONC. (mg/L)	EXPERIMENTAL PROCEDURES
Naphthalene acetic acid	NAA	0.1 0.5	Shoot formation in tobacco callus formation in tobacco
Benzyl amino purine	BAP	0.5 0.1	Shoot formation in tobacco callus formation in tobacco
2,4-Dichlorophenoxy-acetic acid	2,4-D	0.5	Induction of <i>mas</i> promoter
Zeatin	-	1	Shoot formation in tomato

7.2 Radioactive materials

The radioactive materials (Table 6) were purchased from Amersham Corporation (Arlington Heights, IL) and New England Nuclear (Boston, MA).

Table 6. Radioisotopes

NAME	ACTIVITY	PURPOSE
[α - ³² P]dATP	3000 Ci/mmol (250 μ Ci)	DNA sequencing
[λ - ³² P]ATP	6000 Ci/mmol (5 mCi)	end labelling
[α - ³² P]UTP	3000 Ci/mmol (250 μ Ci)	transcription
[α - ³⁵ S]dATP	3000 Ci/mmol (250 μ Ci)	DNA sequencing

7.3 Enzymes and molecular weight markers

Restriction endonucleases, T4-DNA ligase, T4 polynucleotide kinase, Klenow enzyme, calf intestine alkaline phosphatase (CIP), reverse transcriptase, T4-DNA polymerase, S1 nuclease and exonuclease III were purchased from Boehringer Mannheim Biochemicals, Canada (Laval, Quebec) or Bethesda Research Laboratories (BRL, Gaithersburg, MD). Sequenase was obtained from US Biochemicals Corp. (Cleveland, OH). Taq DNA polymerase was supplied by BRL. RNase, proteinase K and lysozyme were purchased from Sigma Chemical Company (St. Louis, MO). Ribonucleoside triphosphates (ATP, CTP, GTP, TTP), deoxyribonucleoside triphosphates (dATP, dCTP, dGTP, dTTP), and dideoxyribonucleoside triphosphates (ddATP, ddCTP, ddGTP, ddTTP) were bought from Boehringer Mannheim.

7.4 Molecular weight markers

For estimating the size of DNA fragments, a HindIII digest of bacteriophage λ DNA was obtained from Boehringer Mannheim; 123 bp ladders were purchased from BRL.

For estimating the molecular weight of proteins, broad range and low range molecular weight markers were purchased from Bio-Rad Laboratories, Inc. (Melville, NY).

7.5 Enzyme Kits

Gene Amp DNA amplification reagent kit (for PCR) was purchased from Perkin Elmer Cetus (Norwalk, CT). Ampli Taq cycle sequencing kit was bought from Perkin Elmer Cetus. DNA labeling and detection kit was obtained from Boehringer Mannheim. The DIG nucleic acid detection kit was purchased from Boehringer Mannheim. Sequenase Version 2.0 sequencing kit was obtained from United States Biochemical Corporation (Cleveland OH). GeneClean II kit for isolation of DNA fragments was ordered from Bio 101, Inc. (La Jolla, CA).

8. Transfer membranes and autoradiography supplies

Hybond-N transfer membranes for Southern hybridizations were from Amersham. MagnaGraph nylon transfer membranes for Southern hybridization were from MSI (Westboro, MA). Zeta-Probe blotting membranes for Northern hybridization were ordered from Bio-Rad. Immobilon-P transfer membranes for protein (Western) blotting were from Millipore Corporation, (Bedford, MA). Kodak Diagnostic films (Rochester, NY) were used for detection of radioactive signals during isotope mediated experiments.

9. Growth Media

9.1 *E. coli*

Luria broth (LB): 10 g tryptone, 5 g yeast extract, 10 g NaCl per liter, adjusted to

pH 7.0 with NaOH. LB plates consisted of LB solidified with 15 g agar per liter.

YEB+Mg²⁺ medium: 5 g beef extract, 1 g yeast extract, 1 g peptone, 5 g sucrose, 0.1 g MgSO₄·7H₂O per liter, adjusted to pH 7.3.

2 x YT Medium: 1.6% tryptone, 1% yeast extract, 0.5% NaCl, pH 7.0

NZY Plates: 1% NZY amine, 0.5% yeast extract, 0.5% NaCl, pH 7.4, 1.2% agar

NZYMM Broth: NZY medium with 0.2% maltose, 0.1 M MgSO₄

TSS: LB broth with 10% (w/v) PEG (MW 3350 or 8000), 5% (v/v) DMSO, 50 mg Mg²⁺ (MgSO₄ or MgCl₂), pH 6.5

SOB: 2% Bacto tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄

SOC: SOB with 20 mM glucose

9.2 Tobacco plants

Medium for seed germination:

1 x MS medium (JRH Biosciences) 3% sucrose, kanamycin was added to a final concentration of 100 mg/L as required for plasmid selection.

Medium for Agrobacterium mediated leaf-disc transformation (MSO):

1 x MS salt, 3% sucrose, 1 x B5 vitamins, pH 5.8, 0.014 M acetosyringone was added prior to transformation.

Shooting medium:

During the shooting process, leaf discs were incubated in MS medium (Murashige minimal organic medium, JRH Biosciences, Lenexa, KS) containing 2% sucrose, 0.22% gelrite, 300 mg/L claforan, 100 mg/L kanamycin and the plant hormones NAA

and BAP, at concentrations of 0.1 mg/L and 0.5 mg/L, respectively.

Rooting medium:

Shooting medium without plant hormone supplementation.

Callus formation medium:

For initiation of callus formation, leaf or stem tissues were placed in MS medium containing the auxin 2,4-D at 3 mg/L. Newly formed calli were maintained initially in MS medium supplemented with 0.5 mg/L 2,4-D then transferred MS medium supplemented with 2 mg/L NAA and 0.1 mg/L BAP.

9.3 Tomato plants

Medium for seed germination:

1/2 MSO (2.3 g Gibco M8 salts, 3% sucrose, 1 x B5 vitamins),
0.22% gelrite, adjusted to pH 5.8.

Shooting medium (D1):

1 x MS salts (Sigma, St. Louis, MO), 3% glucose, 1 x B5 vitamins, 1 mg/L zeatin,
0.22% gelrite, adjusted to pH 5.8. Kanamycin (100 mg/L) was added as required.

Rooting medium:

1 x MS medium (JRH), 2% glucose, 0.22% gelrite

10. Buffers and Solutions

The most buffers and solutions used in Methods are listed below; commonly used solutions not described below can be found in Sambrook et al., 1989.

10.1 Enzyme reaction buffers

10 x Ligation Buffer:

250 mM Tris-HCl, pH 7.6, 50 mM MgCl₂, 25% PEG 8000,
5 mM ATP, 5 mM DTT

10 x Klenow Buffer:

0.5 M Tris-HCl, pH 7.2, 0.1 M MgSO₄, 1 mM DTT,
500 µg/ml BSA fraction V

10 x T4 Polymerase Buffer:

0.33 M Tris-acetate, pH 7.9, 0.66 M K-acetate,
0.1 M Mg-acetate, 5 mM DTT, 1 mg/ml BSA

10 x Exo III Buffer:

0.66 M Tris-HCl, pH 8.0, 6.6 mM MgCl₂

10.2 Electrophoresis buffers

TBE Buffer:

0.09 M Tris-borate, 0.02 M EDTA, pH 8.0, 0.1 µg/ml ethidium bromide

DNA Loading Buffer:

40% glycerol, 1 mM EDTA, 0.1% (w/v) xylene cyanol,
0.1% (w/v) bromophenol blue

Tris-Glycine Buffer:

0.02 M Tris-HCl, 0.2 M glycine, 0.1% SDS

10.3 Buffers for DNA and RNA isolation

Lysozyme-SMM:

250 µg/ml lysozyme, 0.5 M sucrose, 20 mM maleic acid, 20 mM MgCl₂

TNSB Buffer:

0.1 M Tris-HCl, pH 8.0, 0.1 M NaCl, 0.5% SDS,

0.5% bovine serum albumin (fraction V)

SMM:

0.5 M sucrose, 20 mM maleic acid, 20 mM MgCl_2

Solution I:

50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0,

250 $\mu\text{g/ml}$ lysozyme

Solution II:

0.2 N NaOH, 0.1% SDS

Solution III:

3 M potassium acetate, pH 4.8

TE Buffer:

10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0

PEG-NaCl Solution:

30% PEG 8000, 1.6 M NaCl

High Salt Buffer:

5.8 g NH_4OAc , 1.0 mg bromophenol blue in 10 ml

CTAB Isolation Buffer:

2% (w/v) CTAB (hexadecyltrimethylammonium bromide), 1.4 M NaCl, 0.2% (v/v)

2-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0

RNA Extraction Buffer for E. coli:

50 mM Tris-HCl, pH 7.8, 0.1 M NaCl

RNA Extraction Buffer for Plants:

100 mM LiCl, 1% SDS, 100 mM Tris, pH 9.0, 1 mM EDTA

Acridine Orange:

10 mg/ml in H_2O , stored in the dark at 4°C

10.4 Buffers for DNA and RNA analysis

Denaturation Solution:

1.5 M NaCl, 0.5 M NaOH

Neutralization Solution:

1.0 M Tris-HCl, pH 7.0, 1.5 M NaCl

20 x SSPE:

175.3 g NaCl, 27.6 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 7.4 g EDTA, pH 7.4 per liter

50 x Denhardt's:

1% Ficoll (Type 400, Pharmacia), 1% polyvinyl pyrrolidone,

1% bovine serum albumin (Fraction V)

Depurination Buffer: 0.25 N HCl

10 x SSC: 1.5 M NaCl, 0.15 M Sodium citrate, pH 7.5

Hybridization Solution for Southern hybridization:

50% formamide, 5 mM EDTA, 50 mM NaHPO_4 , 0.9 M NaCl, 10 x Denhardt's,

250 $\mu\text{g/ml}$ salmon sperm DNA, 0.1% SDS

Hybridization Solution for plaque hybridization:

6 x SSPE, 5 x Denhardt's, 0.5% SDS, 10 $\mu\text{g/ml}$ salmon sperm denatured DNA

Washing Buffer:

50 mM NaCl, 20 mM NaHPO_4 , 1 mM EDTA, 0.1% SDS

Hybridization Solution for Northern Hybridization:

6 x SSPE, 7% SDS, 5 x Denhardt's

Buffer I for Primer Extension:

140 mM β -mercaptoethanol, 100 mM Tris-HCl, pH 8.3, 0.7 M KCl

Buffer II for primer Extension:

133 mM Tris-HCl, pH 8.3, 16 mM MgCl_2 , 1.3 mM dNTPs

10.5 Buffers for protein analysis

Protein Sample Buffer:

0.05 M Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 2 mg/ml bromophenol blue,
1% β -mercaptoethanol

Homogenization Buffer:

50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 1mM PMSF,
1% Triton X-100

Protein Staining Solution:

45% methanol, 10% glacial acetic acid, 0.2% Coomassie brilliant blue

PBS Buffer:

4% NaCl, 0.1% KCl, 1% Na_2HPO_4 , 0.2% KH_2PO_4

Transfer Buffer:

25 mM Tris, 150 mM glycine, 20% methanol

TBST Buffer:

10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, pH 8.0

Alkaline Phosphate Buffer:

10 mM Tris-HCl, 100 mM NaCl, 3.5 mM MgCl_2 , pH 9.5

10.6 Solutions for bacterial transformation

TB Transformation Buffer:

10 mM Pipes, 55 mM MnCl_2 , 15 mM CaCl_2 , 250 mM KCl.

All the components except for MnCl_2 were dissolved in water and pH was adjusted to 6.7 with KOH. MnCl_2 was added after. The solution was sterilized by filtration through a 0.45 μm filter unit and stored at 4°C.

EB: 0.625 M sucrose, 1 mM MgCl_2

SM: 0.58% NaCl, 0.2% MgSO₄, 0.02 M Tris-HCl, pH 7.5, 0.01% gelrite

STE: 0.1 M NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA

10.7 Buffers for luciferase assay

Luciferase Assay Buffer:

50 mM sodium phosphate, pH 7.0, 0.4 M sucrose, 50 mM β-mercaptoethanol

FMN Solution:

1 mM FMN, 200 mM Tricine buffer, pH 7.0

10.8 Solutions for DNA sequencing

4 x NT Buffer:

0.2 M Tris-HCl, pH 7.8, 20 mM MgCl₂, 10 mM DTT, 0.2 mg/ml BSA

S1 Stop Mix:

0.3 M Trizma base, 0.05 M EDTA

Sequencing Loading Buffer:

80% (v/v) deionized formamide, 10 mM NaOH, 1 mM EDTA,

0.1% (w/v) xylene cyanol, 0.1% (w/v) bromophenol blue

5 x Sequencing Buffer:

0.2 M Tris-HCl, pH 7.5, 0.1 M MgCl₂, 0.25 M NaCl

1 x Labelling Mix:

1.5 μM dGTP, 1.5 μM dCTP, 1.5 μM dTTP

Table 7. Sequencing Mixes for Klenow enzyme

ddA mix	dATP	0.5 mM	5.0 μ l
	dGTP	10 mM	4.5 μ l
	dCTP	10 mM	4.3 μ l
	dTTP	10 mM	4.3 μ l
	ddATP	10 mM	3.5 μ l
	dH ₂ O		78.4 μ l
ddC mix	dATP	0.5 mM	2.0 μ l
	dGTP	10 mM	4.3 μ l
	dCTP	0.5 mM	30.0 μ l
	dTTP	10 mM	4.3 μ l
	ddCTP	10 mM	10.0 μ l
	dH ₂ O		49.2 μ l
ddG mix	dATP	0.5 mM	2.0 μ l
	dGTP	0.5 mM	15.0 μ l
	dCTP	10 mM	4.3 μ l
	dTTP	10 mM	4.3 μ l
	ddGTP	10 mM	10.0 μ l
	dH ₂ O		64.4 μ l
ddT mix	dATP	0.5 mM	2.0 μ l
	dGTP	10 mM	4.3 μ l
	dCTP	10 mM	4.3 μ l
	dTTP	0.5 mM	4.0 μ l
	ddTTP	10 mM	10.0 μ l
	dH ₂ O		73.2 μ l
Chase mix	dATP	10 mM	25 μ l
	dGTP	10 mM	25 μ l
	dCTP	10 mM	25 μ l
	dTTP	10 mM	25 μ l
	dH ₂ O		100 μ l

Table 8. Sequencing Mixes for Sequenase Reactions

ddG mix	dGTP	0.5 mM	32.0 μ l
	dATP	0.5 mM	32.0 μ l
	dTTP	0.5 mM	32.0 μ l
	dCTP	0.5 mM	32.0 μ l
	ddGTP	0.5 mM	3.2 μ l
	NaCl	5.0 M	2.0 μ l
	dH ₂ O		66.8 μ l
ddA mix	dGTP	0.5 mM	32.0 μ l
	dATP	0.5 mM	32.0 μ l
	dTTP	0.5 mM	32.0 μ l
	dCTP	0.5 mM	32.0 μ l
	ddATP	0.5 mM	3.2 μ l
	NaCl	5.0 M	2.0 μ l
	dH ₂ O		66.8 μ l
ddT mix	dGTP	0.5 mM	32.0 μ l
	dATP	0.5 mM	32.0 μ l
	dTTP	0.5 mM	32.0 μ l
	dCTP	0.5 mM	32.0 μ l
	ddTTP	0.5 mM	3.2 μ l
	NaCl	5.0 M	2.0 μ l
	dH ₂ O		66.8 μ l
ddC mix	dGTP	0.5 mM	32.0 μ l
	dATP	0.5 mM	32.0 μ l
	dTTP	0.5 mM	32.0 μ l
	dCTP	0.5 mM	32.0 μ l
	ddCTP	0.5 mM	3.2 μ l
	NaCl	5.0 M	2.0 μ l
	dH ₂ O		66.8 μ l

METHODS

1. Techniques Used in Molecular Cloning

If an enzyme reaction used in this project is not described below, please refer to Sambrook et al. (1989).

1.1 Restriction endonuclease digestion of DNA

Each 20 µl reaction contained 1-2 µg of plasmid DNA, 2 µl of 10 x restriction endonuclease buffer (provided by the manufacturer), 1-2 units restriction enzyme, and double-distilled water (ddH₂O) to attain the final volume in a 1.5 ml eppendorf tube. Reactions were incubated at the optimum temperature for enzyme activity (most require 37°C) for one hour. Reactions were terminated either by heating the tube for 10 minutes at 70°C or, for heat-resistant enzymes, by the addition of 1/10 volume of 200 mM EDTA (pH 8.0). Conditions for bacteriophage λ DNA required digestion were the same but needed a longer incubation (2-3 hours). Digestion of large amounts of bacterial or plant genomic DNA (10-20 µg) in a 40 µl volume required highly concentrated restriction enzymes (30-50 units/µl) and an incubation of 4-16 hours at the appropriate temperature for the enzyme.

1.2 Dephosphorylation of 5' termini of DNA

The removal of 5' terminal phosphate groups from linearized plasmid DNA or DNA fragments using calf intestinal alkaline phosphatase (CIP) prevents self-ligation. Each 20 µl reaction of 0.1-1 µg linearized DNA fragments was carried out in CIP buffer (provided by the manufacturer) with 10 units of CIP for 30 minutes at 37°C in a 1.5 ml eppendorf tube. The reaction was terminated by heating the tube for 15 minutes at 65°C. Following phenol-

chloroform extraction of the enzyme (Methods 1.7), an ethanol precipitation of DNA was carried out (Methods 1.8).

1.3 Ligation of DNA fragments and vectors

Each 20-50 μ l reaction was performed in 5 x ligation buffer (Materials 10.1) with two units of T4 DNA ligase in a 1.5 ml eppendorf tube at room temperature (22°C) for 4-16 hours. The molar ratios of fragment to vector DNA were at least 3:1 (10:1 when DNA linkers were inserted instead of DNA fragments).

1.4 Repair of 5' protruding end of DNA fragments by Klenow enzyme

The Klenow (large) fragment of DNA polymerase I converts the single-stranded 5' protruding ends of DNA generated by restriction endonucleases into blunt double-stranded ends by synthesizing a complementary DNA strand. Each 20 μ l reaction contained 0.5-1 μ g DNA fragments, 2 μ l of 10 x Klenow buffer, 1 μ l of 2 mM dNTPs (2 mM dATP, 2 mM dCTP, 2 mM dGTP, and 2 mM dTTP) and two units of Klenow enzyme in a 1.5 ml Eppendorf tube. Following the 30 minute incubation at 37°C, the enzyme was inactivated by the addition of 1/10 volume of 200 mM EDTA (pH 8.0) and incubated for 10 minutes at 65°C.

1.5 Repair of 3' protruding ends of DNA fragments by T4 DNA polymerase

In contrast to Klenow, T4 DNA polymerase converts the single-stranded 3' protruding end of DNA generated by some restriction endonucleases into blunt double-stranded ends by removing the single-stranded DNA. The reaction was carried out in two steps. First, a 19 μ l reaction containing 500 ng DNA fragments, 2 μ l 10 x T4 polymerase buffer (Materials 10.1), 2 units of T4 DNA polymerase, and double-distilled water in a 19 μ l in a 1.5 ml

ependorf tube. The mixture was incubated for 2 minutes at 37°C. Second, 1 µl of 2 mM dNTPs was added and the reaction incubated for an additional 15 minutes. The reaction was terminated by the addition of 1/10 volume of 200 mM EDTA, pH 8.0. The volume of the reaction was adjusted to 100 µl volume with double-distilled water, enzyme extracted once with phenol-chloroform (Methods 1.7), and the DNA fragments precipitated with ethanol (Methods 1.8).

1.6 Phosphorylation of 5' DNA ends by T4 polynucleotide kinase

There were two important applications for the use of T4 polynucleotide kinase to add 5' phosphate groups to the 5' ends of DNA molecules: (1) "end-labeling" of 5' termini of DNA fragments with radioactive phosphate as probes for hybridization and primer extension reactions and (2) phosphorylation of molecular cloning linkers and PCR fragments for ligation. Each 10 µl end-labeling reaction was carried out with 10 pmoles of oligonucleotides (to be labeled), 1 µl 10 x kinase buffer, 1 µl [γ -³²P]ATP (25 pmoles) and 1 µl of T4 polynucleotide kinase (8 units/µl). Following incubation for 30 minutes at 37°C, the reaction was extracted once with phenol-chloroform and once with chloroform to remove the enzyme. For maximum recovery, the end-labeled oligonucleotides were precipitated with ethanol at -20°C overnight. If an overnight precipitation was not practical, the oligonucleotides were precipitated instead by the addition of 1/3 volume of 7.5 M ammonium acetate and 4 volumes of 100% ethanol followed by incubation for 20 minutes at -20°C. When PCR products or DNA cloning linkers needed to be phosphorylated, 50 µl reactions contained 0.5 µg DNA, 1 µl of 10 mM ATP, 5 µl of 10 x kinase buffer and 1 µl of T4 polynucleotide kinase. Following incubation at 37°C for 30 minutes, the enzyme was removed by phenol extraction and precipitated with ethanol as described (Methods 1.8).

1.7 Phenol preparation and extraction of proteins from DNA

Water-saturated phenol was prepared as follows. After 500 g of phenol crystal (GIBCO, BRL) was melted by warming in a 65°C water bath, about 500 ml of double-distilled water was added to the melted phenol, stirred with a magnetic bar for 10 minutes at room temperature, then kept at room temperature overnight. The water phase was removed the next morning, and the same amount of water was added and stirred once more. After the two phases were separated, most of the water on top was removed. About 1 cm of water was left on top of the phenol to prevent the saturated phenol from oxidizing. Water-saturated phenol was stored at 4°C for a few months and used for the purification of RNA.

Tris-saturated phenol was prepared as follows: 50 g of phenol was melted in a 65°C water bath then mixed with 50 mg of 8-hydroxyquinoline and 50 ml of 0.5 M Tris (no pH adjustment) and stirred (by magnetic bar) for 10 minutes at room temperature. The upper aqueous phase was aspirated and discarded. The procedure was repeated by addition of 50 ml of 0.5 M Tris (pH 8.0) until the phenol phase reached a pH of 7.8-8.0. The Tris-saturated phenol was then stored at 4°C for purification of DNA.

Phenol-chloroform solution was made by mixing equal volumes of Tris-saturated phenol and chloroform: isoamyl alcohol (24:1). DNA samples were extracted with an equal volume of phenol-chloroform solution, then precipitated with 1/10 volume of 3 M NaOAc (pH 5.2) and 2 volumes of 100% ethanol.

1.8 DNA precipitation

The DNA solution was adjusted to a minimum volume of 50 µl with double-distilled water in an eppendorf tube. After the addition of 1/10 volume of 3 M NaOAc (pH 5.2) and 2 volumes of 100% ethanol, the mixture was placed at -20°C for 30 minutes, then centrifuged at 10,000 rpm for 5 minutes to collect the precipitated DNA. The pellets were

then washed with 70% ethanol and dried in a speed vac for 5 minutes. The DNA was then resuspended in the desired volume of ddH₂O.

1.9 Truncation of DNA Fragments using ExoIII

Unidirectional deletions (truncations) of DNA fragments were produced using exonuclease III (Exo III) activity (Henikoff, 1987). This was a two step process.

First, a plasmid (1 µg) containing the DNA fragment of interest was linearized with an appropriate restriction enzyme to create a 5' protruding end on one side of the insert. After the reaction was completed, the DNA was precipitated and dissolved in 10 µl of water. The 5' protruding ends were filled in (blunted) with 2 units of Klenow enzyme, 5 µl of 4 x NT buffer, 3 µl of 3 x dNTPs (dCTP, dGTP, dTTP, 1 mM each), and 1 µl of thio-dATP. The incorporation of thio-dATP created a point mutation which inhibited the 3'→5' editing function of ExoIII. The fill in reactions were carried out at 37°C for 10 minutes, and DNA was precipitated as described in Methods 1.8. After centrifugation, the linearized plasmid DNA was resuspended in water for cleavage by a second restriction endonuclease. The cleavage site of this restriction enzyme must be located inside the first cleavage site going toward the insert; this restriction enzyme must also produce 5' protruding ends. After the second digest was completed, the plasmid DNA pellet was resuspended in 16 µl of water.

Second, the deletion reactions were carried out by the addition of 2 µl of 10 x Exo III buffer and 2 µl of exonuclease III (175 units/µl) for 0-10 minutes at 37°C. During this time, 2 µl of the content was removed into a clean tube each minute (or 4 µl every 2 minutes). The tubes contained 6 µl of S1 mix (1 x S1 buffer and 3 units of S1 nuclease). S1 nuclease immediately removed the strand of DNA which was not truncated by ExoIII. The reaction was complete after 30 minutes at room temperature. S1 nuclease reactions were stopped by the addition of 1 µl S1 stop mix, and incubated at 70°C for 10 minutes. The

results of truncations were checked by loading part of the reaction solutions on agarose gels to estimate the size of the DNA. Meanwhile, the ends of the DNA fragments were repaired by Klenow enzyme and ligated (Methods 1.3). The recircularized plasmids containing a series of truncated inserts were transformed into *E. coli* MV1193 cells. Individual plasmid constructs from transformants were analyzed by restriction enzyme mapping and gel electrophoresis.

1.10 Primer design

Oligonucleotide primers for DNA sequencing and subcloning were designed based on G+C content. For DNA sequencing, the primers were chosen as 16-18 bases long with a G+C content higher than 60%. Primer design tried to avoid possible secondary structure within the sequence. Primers for PCR and subcloning were between 20-28 bases. When restriction enzyme cutting sites were required on both sides of the primers, the design was based on the cleavage ability of a restriction endonuclease within a few bases at the ends of a DNA fragment. The information was provided by manufactures, such as New England Biolabs and Stratagene.

1.11 Polymerase chain reaction (PCR)

PCR, an *in vitro* method of nucleic acid synthesis, was utilized to amplify DNA fragments from *B. subtilis* genomic DNA. PCR employs two oligonucleotide primers that flank the DNA fragment to be amplified and repeated cycles of heat denaturation of DNA, annealing of primers to their complementary sequences, and extension of the annealed primers with DNA polymerase. Since the extension products themselves are also complementary to and capable of binding primers, successive cycles of amplification occur exponentially. The number of nucleic acid molecules produced from the first two strands

is equal to 2^n , where n is the number of cycles of amplification performed.

Oligonucleotides synthesized by DNA synthesizer (ABI, 390B) were dissolved in double-distilled water. Aliquots of samples (10 μ g) were loaded on a 20% polyacrylamide gel and the electrophoresis was performed as shown in Methods 2.2. After the electrophoresis was completed, the DNA was visualized in the gel directly under the UV light without ethidium bromide staining, and DNA containing gel was removed using a razor blade. The gel slices were placed in 0.3 M NaAc (pH 5.2) and shaken at room temperature for 3 hours to elute oligonucleotides from the polyacrylamide gel slices. The supernatant was extracted with phenol-chloroform (1:1) and oligonucleotides which were precipitated with ethanol were ready for the polymerase chain reaction.

The PCR reactions were set up in 0.5 ml eppendorf tubes at a total volume of 100 μ l. A standard reaction mix contains the following reagents: 4 μ l of 5 mM dNTPs, 1 μ M primer #1, 1 μ M primer #2, 10 ng template DNA (when plasmids were used, 10-20 μ g template DNA when genomic DNA was used), 2-3 units of Taq DNA polymerase, 10 μ l of 10 x Taq DNA polymerase buffer, and 1.5 mM $MgCl_2$ (if not included in the polymerase buffer system). Water was used to adjust the volume to 100 μ l. The mixture was then overlaid with 100 μ l of mineral oil. The reaction tubes were put in a DNA thermal cycler (Perkin Elmer Cetus 4800 or 9600) and PCR was programmed for 25-35 cycles using the following temperature profile: 94°C, 1 minute (for denaturation); 60°C, 1 minute (for annealing); 72°C, 2 minutes (for primer extension). Temperature and incubation period were varied depending on templates and products. When primers were as short as 16-18 bases, the annealing temperature was reduced to 55°C; when PCR products were shorter than 1 Kb, the extension period was decreased to 1 minute. Reactions were stopped by chilling to 4°C using a delay file and product size was analyzed on agarose or polyacrylamide gels.

2. Gel Electrophoresis

2.1 Agarose gels for separation of DNA or RNA

Agarose gels were prepared by adding 0.7 g of agarose (ultra pure, IBI) to 100 ml of TBE buffer (Materials 10.2) in an erlenmeyer flask (0.7% agarose gel). When DNA fragments smaller than 0.5 kb were to be visualized, a 1.0% agarose gel was prepared. The agarose was heated using a microwave oven until well-dissolved, cooled down to 55°C, then poured onto a gel apparatus to form a gel of 5-7 mm thickness. DNA fragments were mixed with 1/10 volume DNA loading buffer (Materials 10.3) and loaded under TBE buffer into the wells of the gel. The electrophoresis of DNA fragments in agarose gels was carried out in TBE buffer at room temperature either overnight (12-16 hours) at 35 Volts or 30 minutes to 1 hour at 100 Volts. Ethidium bromide (10 µg/ml) was added to the agarose solution during gel preparation. DNA fragments were visualized using a transilluminator box (UV, wavelength 390 nm) in a dark room and photographed using a Polaroid camera with Polaroid Type 57 film. Due to the susceptibility of RNA to degradation, the analysis of RNA samples on agarose gels was carried out in a RNase free system. The buffer system of electrophoresis was treated with DEPC (diethyl pyrocarbonate) overnight to inactivate RNase and autoclaved prior to the electrophoresis; the gel apparatus was incubated with 0.2 N NaOH overnight, then rinsed with DEPC-treated ddH₂O.

2.2 Polyacrylamide gels for DNA sequence analysis, separation of oligonucleotides, and separation of small DNA fragments

DNA sequence analysis was performed in 6% polyacrylamide gels prepared as follows. 11.25 ml of 40% acrylamide solution (acrylamide: bis-acrylamide = 38 : 2), 7.5 ml of 10 x TBE buffer, 37.5 g of urea (sequencing grade) were mixed and adjusted to 75 ml by addition of water in a 100 ml cylinder. The mixture was incubated in a warm water bath until

all the urea was dissolved. The solution was cooled to room temperature followed by the addition of 250 μ l of ammonium persulfate (10%) and 25 μ l of TEMED (Bio-Rad). This solution was poured immediately, a gel comb inserted to form wells for loading reactions, and the gel was allowed to polymerize at room temperature. A 15% polyacrylamide gel was prepared for oligonucleotide detections and a 5% polyacrylamide gel formed for analyzing DNA products during primer extension experiments.

2.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) for separation of proteins

Total protein samples from bacterial cells or plant tissues were prepared by different methods. Aliquots (100 μ l) of fresh bacterial culture (at a concentration of 1-1.5 OD₆₀₀) were concentrated by centrifugation and resuspended in 50 μ l of protein sample buffer (Materials 10.5). After vigorous vortex mixing and boiling for 3 minutes at 100°C, the solutions were sonicated briefly to break large DNA fragments to reduce viscosity. Samples of 2-15 μ l were loaded per well for protein analysis by polyacrylamide gel electrophoresis. In contrast, plant leaf or fruit tissues were ground in homogenization buffer (Materials 10.5) in a mortar with a pestle. The mixture was transferred to a tube and centrifuged for 5 minutes at 2,000 g. Supernatants were removed to clean tubes and 1/4 volume of protein sample buffer was added. All the processes were carried out at room temperature.

Total protein samples were separated by vertical SDS-PAGE, mainly in 10-25% gradient gels (1 mm) or 7.5%-20% gradient gels (3 mm). The separating gels were prepared from a 40% acrylamide stock solution (39% : 1% = acrylamide : bis-acrylamide) with Tris-HCl (pH 8.8) at a final concentration of 0.375 M Tris-HCl and SDS at final concentration of 0.1%. To initiate gel polymerization, ammonium persulfate (0.025%) and TEMED (0.025%) were added just before pouring the gel solution into the gel apparatus. The loading level was controlled so that a 3 cm space remained at the top to accommodate the stacking

gel which received the protein samples. After the separating gel polymerized (1-3 hours), a stacking gel containing 4.75% acrylamide in 0.125 M Tris-HCl, pH 6.8, 0.1% SDS was added, a gel comb inserted, and polymerization allowed to continue for 1 hour. Protein samples were boiled for 3 minutes prior to loading under buffer. The electrophoresis was carried out in Tris-glycine buffer (Materials 10.5) using the Bio-Rad PROTEAN II xi 2-D Cell gel apparatus for 3-4 hours at 70 mA, or 16 hours at 35 mA for 3 mm gels, 35 mA for 3-4 hours or 10 mA for 16 hours for 1 mm gels. After completion of the electrophoresis, the gel was either stained in protein staining solution (Materials 10.5), or transferred to membranes for immunoblot analysis.

3. Transformation

3.1 *One-step transformation of E. coli*

This time-saving but lower efficiency protocol (Chung et al., 1989) was suitable for transformation with specific plasmid constructs but was not used for subcloning mixtures. *E. coli* cells (JM101, JM109, HB101, DH5 α) were grown in LB broth to early log phase culture density (OD₆₀₀ 0.3-0.4) and collected by centrifugation at 1,000 x g for 10 minutes at 4°C. The cell pellet was resuspended in 1/10 of volume of ice-cold transformation and storage solution TSS (Materials 10.6). Cell aliquots (0.1 ml) were transferred into 15 ml precooled polypropylene tubes and mixed with 1 μ l (1-100 ng) of plasmid DNA, then incubated for 30 minutes on ice. LB broth (0.9 ml) was added after the incubation was completed, and the cell suspension was shaken at 37°C for 1 hour to allow expression of the antibiotic-resistance marker gene. Each 0.1 ml aliquot was spread on the surface of an LB selective medium plate (containing the appropriate antibiotic for selecting the plasmid construct). To facilitate carrying out transformation experiments, competent cells in TSS were frozen in liquid nitrogen and stored at -70°C. When needed, frozen cells were thawed

on ice and used immediately. The transformation efficiency obtained with this procedure was approximately 10^6 to 10^7 cfu (colony-forming units) per μg plasmid DNA.

3.2 High efficiency transformation of *E. coli*

Competent cells were prepared from *E. coli* (DH5 α , JM109, HB101) according to the method of Inoue et al. (1990). 100-fold dilutions of overnight cultures (37°C) were inoculated into 250 ml of SOB medium (Materials 9.1) in a 2-liter flask and shaken vigorously (250 rpm) for 8-12 hours at room temperature (22°C) until culture absorbance reached $\text{OD}_{600} = 0.6$. At that time, the suspension was placed on ice for 10 minutes before centrifugation at 2,500 g for 10 minutes at 4°C. The cell pellet(s) were resuspended in 80 ml of ice-cold TB transformation buffer (Materials 10.6), incubated on ice for 10 minutes, and centrifuged for a second time. The cell pellet(s) were gently mixed with 20 ml of cold TB buffer and DMSO added to 0.7% with gentle swirling. After 10 minutes incubation in an ice bath, the cell suspension was dispensed in 100 μl aliquotes and quickly immersed in liquid nitrogen. The frozen competent cells were stored in -70°C the competency maintained for up to six months.

Transformation was carried out with frozen competent cells, allowed to thaw briefly at room temperature then placed on ice. For each transformation, 1-5 μl of the plasmid (10 ng-100 ng) solution was added to 100 μl competent cells and incubated on ice for 30 minutes in a 1.5 ml eppendorf tube. Tubes were then heat-pulsed at 42°C for 1 minute and returned to ice. After the addition of 0.8 ml of SOC medium (Materials 9.1), the mixture was transferred to a 10 ml polypropylene tube and shaken at 37°C for 1 hour. A 0.1 ml aliquot of the suspension was then spread onto selective agar plates (containing the appropriate antibiotic) and colonies appeared after 12 hours incubation at 37°C. This method yielded a transformation efficiency of 10^8 to 10^9 cfu per μg DNA.

3.3 Transformation of *A. tumefaciens*

The efficient way to transform plasmid DNA into *Agrobacterium* is by conjugal transfer of the plasmid from *E. coli* to *Agrobacterium*. Since unmodified plasmid DNA from most *E. coli* strains would be recognized by the restriction system of *Agrobacterium*, the plasmid must be donated by *E. coli* S17.1 which can modify the plasmid DNA. For conjugation, *E. coli* S17.1 donor cells were grown overnight on YEB plates with 10 mM Mg^{2+} and 100 mg/L ampicillin at 37°C. At the same time, recipient cells of *Agrobacterium tumefaciens* GV3101(pMP90RK) were grown overnight on YEB plates with 10 mM Mg^{2+} plus 100 mg/L rifampicin, 25 mg/L kanamycin, and 25 mg/L gentamycin at 29°C. For the donor strain, colonies were removed from the plates and resuspended in 5 ml of YEB + Mg^{2+} medium. Cells were collected again by centrifugation and resuspended in 3 ml of YEB + Mg^{2+} medium. The same procedure was repeated to harvest the recipient cells. For each cell suspension, absorbance was measured at OD_{600 nm} using a spectrophotometer. Donor cells (*E. coli* S17.1) and recipient cells (*Agrobacterium tumefaciens* GV3101pMP90RK) were diluted to 6×10^8 cells in a volume of 50-200 μ l and diluted to 2×10^8 cells in a volume of 50-200 μ l, respectively. Conjugation was initiated by mixing together 50-200 μ l of *E. coli* and *Agrobacterium* on several YEB + Mg^{2+} plates. The plates were placed in a 29°C incubator. After 2-3 days of conjugation, cells from these plates were picked up with a sterile inoculation loop and transferred to 10 ml of 10 mM $MgCl_2$. Cells were washed with 10 mM $MgCl_2$ and resuspended in 5 ml of 10 mM $MgCl_2$. Absorbance of the cell suspension was measured at OD_{600 nm} and several dilutions (of 10^7 - 5×10^8 cells) were spread on YEB + Mg^{2+} plates containing Rif (100 mg/L), Km (25 mg/L), Gm (25 mg/L) and Carb (100 mg/L) antibiotic selections. The single colonies of transconjugants were screened several times on selective plates. Finally the plasmid was isolated from the transconjugants. Usually, a large amount of plasmid DNA was difficult to obtain from *Agrobacterium* strains, making

identification of plasmid difficult. This problem was alleviated by back-transformation of the plasmid to an *E. coli* strain (e.g. DH5 α).

3.4 Transformation of tobacco plants

Agrobacterium tumefaciens mediated leaf-disc transformation was employed to transform plants with expression vectors. Successful transformations required well-grown *Agrobacterium* strain and healthy, young-looking plant leaf materials. Young plant leaf tissues were obtained from sterile *N. tabacum* SR1 plants grown in jars in the light room. (The culture solutions and media for this procedure are provided in Materials 9.2). An overnight culture of *A. tumefaciens* strain which contained the plant expression vector was prepared in 10 ml LB broth with antibiotic selections. The cells were collected and the antibiotics were removed by washing the cell pellet with LB medium and finally resuspended in 1 ml of LB broth. A 200 μ l of above *Agrobacterium* suspension was combined with 20 ml of MSO medium followed by addition of 500 μ l of acetosyringone stock solution (0.0148 M in ethanol, stored at -20°C) in a 9 cm diameter culture dish. Under sterilized conditions, leaf discs were excised from sterile SR1 leaves with a cork borer (ϕ 1 cm), and submerged in MSO-*Agrobacterium* culture for 5 minutes. Individual leaf discs were removed with forceps from the *Agrobacterium* solution and placed upside down on MS solid medium plates containing 0.5 mg/L NAA and 0.1 mg/L BAP. The plates were wrapped with aluminum foil and incubated in the light room.

After two days, the leaf discs were moved to MS shooting medium containing 300 mg/L claforan and 100 mg/L kanamycin, and kept in the light room. The leaf discs were transferred to fresh shooting medium at ten day intervals. When the kanamycin resistant tobacco shoots appeared and had grown to about 1 cm, they were cut off from the bleached leaf discs and transferred to tobacco rooting medium containing claforan (300 mg/L) and

kanamycin (100 mg/L) in jars. When root systems were developed, the whole plants were moved to soil and grown in the greenhouse (Methods 17.4).

3.5 Transformation of tomato plants

Agrobacterium mediated cotyledon transformation was used to introduce gene expression vectors into tomato plants. (Media and solutions for this procedure are listed in Materials 9.3). Cotyledons were prepared from sterile tomato *L. esculentum* seeds by germinating them on 1/2 MSO medium for 10-14 days in sterile magenta boxes. When there were no or minimal true leaves present on the seedlings, the cotyledons were collected and used for transformation. To initiate infection by *Agrobacterium*, cotyledons were picked and floated in 5 ml of MSO liquid medium in a 15 x 150 mm petri dish containing 5 µl cultured *Agrobacterium* and 375 µM acetosyringone. Each cotyledon was cross cut into three sections. When about 50 cotyledons were cut (5-15 minutes), they were placed upside down on D1 medium plates without antibiotic selection. After two days incubation in the light room (without foil wrapping), these cotyledons were transferred to plates of D1 medium plates containing kanamycin (100 mg/L) and claforan (300 mg/L). Kanamycin was used for selection of transformants, whereas claforan was used to inhibit the growth of *Agrobacterium*. The plates were sealed with parafilm and incubated in the light room (Methods 17.4). Green calli or shoots were seen at the cut edges within 10 days.

The transformed plant tissues were visualized daily under Zeiss sterile microscope, rotten tissues were removed from time to time, and green calli or shoots along with healthy cotyledons were transferred to fresh solid medium at 10 day intervals. After three to four weeks, shoots with true meristems were cut off cleanly from the cotyledon tissues and transferred to tomato rooting medium in jars with no antibiotic selections. Roots were formed within 10 days. When the roots were developed well, small plantlets were transferred

into very wet soil (Metro-Mix #290) in two-inch pots and covered with plastic bags. They were incubated in greenhouses under low light conditions and plastic bags were slowly removed after five days. The transgenic plants were eventually growing in the greenhouse under normal conditions.

4. Preparation of Plasmid DNA

4.1 *Small - scale isolation*

This procedure provides 1-2 μg of plasmid DNA from 1 ml of bacterial culture in one hour. The DNA is of acceptable quality for restriction endonuclease mapping or enzyme modifications without further purification.

A 3 ml culture of a plasmid-containing bacterial strain was grown overnight in L broth supplemented with the appropriate antibiotics to maintain selection for that plasmid. (Most of the solutions used for plasmid preparations are listed in Materials 10.3). Cells from 1 ml of cell suspension were harvested in a 1.5 ml eppendorf tube by centrifugation for 30 seconds at 10,000 rpm. Cells were resuspended in 100 μl of solution I (for *E. coli* and *A. tumefaciens*) or solution I containing 250 $\mu\text{g}/\text{ml}$ lysozyme (for *B. subtilis*). A 30 minute incubation at 37°C was required for *B. subtilis* but not for *E. coli* or *A. tumefaciens*. To lyse the cells, 200 μl of solution II was added to each tube, mixed well with solution I and incubated on ice for precisely 5 minutes. Lastly, 150 μl of solution III was added, mixed by inverting and incubated on ice for a minimum of 5 minutes. After the mixture was centrifuged in a microfuge for 5 minutes at 10,000 rpm, plasmid DNA containing supernatants were transferred to clean 1.5 ml eppendorf tubes prefilled with 800 μl of 100% ethanol. The mixture was placed at room temperature for 1-5 minutes. The plasmid DNA was then collected by centrifugation. The resulting plasmid pellets were then washed with 70% ethanol and dried in the Speed Vac for 5 minutes. The DNA was finally resuspended

in 20 μl of ddH_2O containing 1 μl RNase (1 $\mu\text{g}/\mu\text{l}$).

4.2 Cesium chloride - ethidium bromide gradient purification

This technique was used to obtain larger quantities of purer plasmid DNA from 500 ml to 1 liter cell cultures. (Note: This procedure uses a large amount of ethidium bromide and has a lower DNA yield than the PEG method which follows.)

Plasmid containing *E. coli* strains were inoculated into 500 ml of L broth containing the appropriate antibiotic. The cells were grown to a density of $\text{OD}_{600} = 1.0$ and collected by centrifugation. An absorbance of 1.0 OD_{600} equals about 8×10^8 cells/ml. The cell pellet was resuspended in 5 ml of solution I containing 250 $\mu\text{g}/\text{ml}$ lysozyme and kept at room temperature for 20 minutes to digest bacterial cell walls. Cells were then lysed by adding 10 ml of freshly made solution II, incubated on ice for 10 minutes, after which, 7.5 ml of solution III was added and incubated further on ice for another 10 minutes. The lysate was then centrifuged at 10,000 rpm for 20 minutes at 4°C and the supernatant carefully poured off into a clean centrifuge tube and precipitated by the addition of 0.6 volumes of isopropanol. The nucleic acids were harvested by centrifugation at 10,000 rpm for 30 minutes at 18°C and the pellet washed with 70% ethanol and dried.

Next the pellets were dissolved in 6 ml of TE buffer and mixed gently with 5.9 g of cesium chloride, until all the CsCl was completely dissolved. The solution was then transferred to a 10 ml ultracentrifuge tube containing 0.3 ml ethidium bromide (10 mg/ml), the remaining space in the tube was filled with paraffin oil and multiple tubes balanced. The centrifugation was carried out at 32,000 rpm for 40 hours at 18°C in a Beckman ultracentrifuge. After completion, DNA bands were visualized by a hand-held UV light and collected by a syringe with the hypodermic needle inserted into the side of the tube just below the band. Ethidium bromide was removed from the DNA samples by repeated

extractions with an equal volume of 1-butanol. Residual cesium chloride was removed by dialysing the samples against TE buffer for 24 hours. DNA concentration and purity were further determined using a Beckman spectrophotometer at UV adsorption of 260 nm and 280 nm.

4.3 Plasmid preparation with PEG

This was the most efficient approach for isolation of large quantities of plasmid DNA from *E. coli* strains. The yield reached as high as 5 mg DNA per liter culture (pBluescript); purity of the plasmid DNA was quantified for DNA sequencing as well as other applications.

Plasmid-containing *E. coli* cells were cultured and treated with solution I, II and III as described in Methods 4.2. After incubation with solution III, the cell suspension was centrifuged at 10,000 rpm at 4°C for 10 minutes to remove fibers and large RNA. The supernatant was transferred into a clean tube, 0.6 volumes of isopropanol was added to precipitate the plasmid DNA for 10 minutes at room temperature. The DNA was pelleted by centrifugation at 10,000 rpm for 30 minutes, and resuspended in 1 ml of TE buffer. To remove RNA, DNA was treated with 5 µl of RNase (10 mg/ml) at 37°C for 30 minutes and precipitated again by the addition of 0.4 volume of PEG-NaCl solution (Materials 10.3) on ice for 30 minutes. The plasmid DNA was collected by centrifugation and resuspended once more in 1 ml of TE buffer. Contaminating PEG and proteins were removed by phenol extraction and plasmid DNA was precipitated once more with 1/4 volume of 10 M NH₄Ac and 2 volumes of 100% ethanol. The plasmid pellet was finally washed with 70% ethanol, dried, and resuspended in 1 ml of TE buffer.

4.4 Purification of DNA fragments from agarose using GeneClean kit

This method provided an efficient way to isolate DNA fragments (< 3 Kb) from

agarose gels. DNA bands were excised from ethidium bromide stained agarose gels with a razor blade. Approximate volume of gel slices was determined by weight (1 g gel equals approximately 1 ml volume) and transferred to polypropylene tubes. Half volumes of TBE Modifier and 4.5 volumes of NaI (both were provided by the manufacturer) per one volume of agarose gel slice were added to the tube to achieve a final concentration of 4 M NaI. Capped tubes containing the agarose gels were placed in a 55°C water bath and incubated for 5 minutes with occasional inverting of the tubes. Glassmilk (silica matrix) 5 µl was added to the solution and vortexed until all the contents were in suspension. If the amount of DNA fragment exceeded 2 µg, an additional 1 µl of Glassmilk was added for each 0.5 µg of DNA. The tubes were kept on ice for 5 minutes, and silica matrix with bound DNA was collected by centrifugation in a microfuge for 10 seconds at room temperature. The resulting DNA pellet was washed three times with 500 µl New Wash (from manufacturer). Bound DNA was eluted from Glassmilk by addition of 20 µl of water followed by incubation at 55°C for 5 minutes. The complete process took about 20 minutes.

4.5 Electroelution of DNA fragments from agarose

For DNA fragments (> 6 Kb), the GeneClean II kit (see above) was much less efficient than electroelution. DNA fragments were separated on a 0.6% agarose gel by electrophoresis. Excess running buffer was removed until no buffer was on top of the gel. A hand-held UV light was used to visualize and localize the stained DNA bands in the gel. A 2 mm U-shaped groove was made by cutting the gel in front of (below) the band. A piece of dialysis tubing was carefully placed into the groove and filled with running buffer. Gel electrophoresis was continued at 150 volts until all the DNA in the UV-visible band ran into the groove. At that time, the current was inverted for 30 seconds to ensure the maximum recovery from the dialysis tubings. DNA containing buffer was then transferred to a tube and

the volume was reduced by extracting water from the solution with 1-butanol. Lastly, the DNA fragments were precipitated under standard procedures (Methods 1.8).

5. Preparation of λ DNA

5.1 Growth of bacteriophage λ

In order to isolate a large amount of λ DNA, phage λ was first amplified in the host cells by mixing 500 μ l of LE392 host cells (see Methods 17.3, lambda growth and purification) with 0.5-5 μ l of multiplied phage solution (see Methods 17.3). The mixture was incubated at 37°C for 20 minutes, 50 ml of NZYM medium was added to the tube, and the culture grown overnight with shaking at 37°C. The next morning, chloroform (0.4 ml) was added to the infected bacterial culture and the flask shaken continuously for 10 minutes to release the phage particles. The culture was then centrifuged for 10 minutes at 10,000 rpm. The supernatant containing the phage particles was transferred into an ultracentrifuge tube, followed by centrifugation at 30,000 rpm for 30 minutes at 4°C. The resulting supernatant was removed and phage particles were suspended in 300 μ l SM by vortexing vigorously.

5.2 Isolation of λ DNA from phage particles

To extract the phage DNA from the capsid protein, phage particles were incubated with 300 μ l of 2 x STE and 60 μ l of proteinase K solution (10 mg/ml) for 10 minutes at 65°C, following centrifugation at 12,000 rpm for 5 minutes at room temperature. The supernatant was collected and the proteins in the supernatant were removed by extraction with phenol-chloroform (Methods 1.7). Bacteriophage DNA was ethanol precipitated, collected by centrifugation, and the resulting λ DNA pellet dissolved in ddH₂O at a final concentration of 0.5 μ g/ μ l.

6. Preparation of Plant Genomic DNA

The method of Doyle et al. (1990) was used to prepare plant genomic DNA from fresh tissues. Genomic DNA was isolated from 1 g of leaf tissue or 3 g of fruit tissue. Cells were broken by grinding the tissues in a mortar with 15 ml of CTAB isolation buffer (Materials 10.3) and sea sand at room temperature. The ground tissue was transferred to a tube and incubated for 30 minutes in 60°C water bath with occasional inverting of the tube. The polysaccharides were extracted from the mixture with equal volume of chloroform and centrifuged at 4,000 rpm for 10 minutes at room temperature. The clear aqueous phase, which contained nucleic acids was transferred to a clean centrifuge tube, and 0.6 volumes of isopropanol were added to precipitate nucleic acids for 5 minutes at room temperature. Nucleic acids were recovered by centrifugation at 4,000 rpm for 5 minutes, and the pellet was washed with 70% ethanol to remove extra salts at room temperature for at least 20 minutes. The pellet was dried, then dissolved in 1 ml of TE containing 10 µl of RNase (10 mg/ml). RNA was digested for 30 minutes at 37°C. DNA was precipitated with 1/4 volume of 10 M NH₄Ac (pH 7.3) and 0.6 volumes of isopropanol at room temperature for 5 minutes. Lastly, the purified DNA was recovered by centrifugation, dried and dissolved in 1 ml of TE.

7. Preparation of total RNA from plant tissues

Fresh plant leaf (3-5 g) or fruit (10-15 g) tissues were sliced into small pieces and frozen immediately in liquid nitrogen. Phenol-extraction buffer (1:1) was freshly prepared and warmed to 90° C in a water bath. The frozen tissues were placed in a cold mortar and ground in liquid nitrogen using a pestle until a fine homogeneous powder was obtained. The powder was scraped into an -70°C prechilled Erlenmeyer flask (250 ml) and immediately mixed with 20-25 ml of phenol-extraction buffer (2 ml buffer/gram fresh weight plant material). The temperature of the mixture (in the flask) was brought up to 25-30°C quickly

by swirling the flask in the 90°C water bath. The flask was then shaken on a gyratory shaker at 300 rpm for 5 minutes at room temperature. To complete the extraction, 10-15 ml of chloroform was added and the flask shaken for 15-30 minutes. The milky suspension was then transferred to a centrifuge tube and centrifuged at 8,000 rpm in the SA600 rotor for 30 minutes at 25°C. The upper phase was transferred to a flask and extracted once more with chloroform. The volume of the upper layer was measured and 1/3 volume of 8 M LiCl was added to precipitate RNA for 8-16 hours at 4°C. RNA was collected by centrifugation at 8,000 rpm for 20 minutes and washed with 80% ethanol. Finally, the dried RNA pellet was dissolved in DEPC-ddH₂O and stored at -70°C.

8. Synthesis of radioactive-labeled probes

8.1 Random primer labeling using DNA fragments directly from low-melting point agarose gels

The DNA fragments to be labeled were purified in low-melting agarose gels by electrophoresis. The desired DNA band was excised from the gel using a razor blade, weighed and placed in water (3 ml/g gel). After heating to 90°C for 2 minutes, the DNA fragment was ready for labeling. The labeling reaction contained approximately 10-20 ng of gel isolated DNA in a 1.5 ml eppendorf tube, 10 µl of 5 x Klenow buffer, 2 µl of hexanucleotide primer (50 A₂₆₀/ml), 3 µl of dCTP, dGTP, dTTP mix (1 mM each), and 1 µl of [α -³²P]dATP (10 µci/µl). The tube was heated to 90°C for 2 minutes to denature the DNA fragments, then held at 37°C for 15 minutes to allow the annealing of primers to the templates. Finally, 2 units of Klenow enzyme was added and DNA synthesis allowed to proceed overnight at room temperature. The labeled DNA probe was purified from unincorporated isotopes by passing the reaction mixture through a Sephadex G-50 column before use.

8.2 Klenow labeling using purified DNA fragments

Purified DNA fragments (100 ng) were mixed with 2.5 μ l of 10 x dextranucleotides (Boehringer Mannheim) in a 1.5 ml eppendorf tube, boiled for 3 minutes to denature the DNA, then transferred to ice immediately to prevent reannealing of DNA strands. Reagents were added in the following sequence: 2.5 μ l of 0.5 mM 3 x dNTP mix (dCTP, dGTP, dTTP), 2.5 μ l of 10 x Klenow buffer, 5 μ l of [α -³²P]dATP, and 4 units of Klenow enzyme. The labeling reaction was carried out at room temperature for 5-16 hours. The reaction mixture was passed through a Sephadex G-50 column and 0.2 ml of fractions were collected. Levels of incorporation (in counts per minute, cpm) were determined by liquid scintillation using a Beckman LS3801 scintillation counter. Labeling was successful if the first peak (higher molecular weight fractions) contained greater than 1×10^8 cpm/ μ g DNA.

8.3 Polynucleotide kinase for end-labeling

Described above in Methods 1.6.

9. Hybridization

9.1 Plaque hybridization

A method modified from Benton and Davis (1977) was used to identify those recombinant bacteriophages which carried DNA sequences of interest in complex cDNA or genomic DNA libraries. All solutions can be found in Materials 10.4.

First, bacteriophage λ plaques were immobilized on nylon membranes (ϕ 82 mm, Schleisher and Schuell). Nylon membranes were numbered with a pencil, and carefully placed on top of the plaque-containing plates so that direct contact was established with the top agar. The position of the membranes was marked by stabbing a syringe through the membranes and into the agar. After 2 minutes, the membranes were peeled off and laid

(bottom side up) on 3 layers of 3MM filter papers saturated with denaturation solution. The membranes were incubated with the filter paper for 3 minutes before they were transferred to another set of filter papers saturated with neutralization solution for another 3 minutes. Incubation with fresh neutralization solution performed for another 3 minutes. Finally, the membranes were placed on filter papers containing 2 x SSPE for 5 minutes, then allowed to dry at room temperature for 1 hour. After the membranes were dried, they were placed on 0.4 N NaOH saturated filter papers for 20-30 minutes, immersed into 5 x SSPE solution for 10-60 seconds, followed by drying on paper towels at room temperature.

Second, prehybridization of the membranes was carried out in hybridization bags using 3 ml hybridization solution with 30 μ l of denatured salmon sperm DNA (10 mg/ml) in a 68°C oven with rotation overnight. The membranes were placed in bags back-to-back so that the DNA containing side was facing the bag. (This was an economical way of dealing with 10-20 membranes at a time.) After prehybridization, the sealed bags were cut open, and 2 ml of hybridization solution containing radioisotope-labeled and heat-denatured DNA probe was added to each bag. Hybridization was allowed to proceed overnight at the same temperature as the prehybridization. After hybridization, membranes were washed 3 times for 30 minutes each with 2 x SSPE at 60°C. To obtain an x-ray exposure, wet membranes were wrapped in polyvinyl-chloride wrap to prevent leakage and drying. Films were exposed for an appropriate amount of time and the films developed, as described in Methods 13.

9.2 Southern hybridization

The DNA transfer techniques of Southern (1975) were used to analyze the location of particular DNA sequences within total genomic DNA. In brief, genomic DNA was digested with one or more restriction enzymes and the resulting fragments were separated according to size by electrophoresis in an agarose gel. The DNA was denatured *in situ* and

transferred from the gel to a solid support (such as nylon membranes). The DNA attached to the filter which was hybridized to radio-labeled DNA and autoradiography was used to locate the position of bands containing DNA sequences complementary to the probe. (Solutions are listed in Materials 10.4.)

The first step is the transfer of genomic DNA fragments from the gel to the nylon membrane. Bacterial, bacteriophage λ , or plant genomic DNA was digested with one or more restriction endonucleases and the DNA fragments were separated in agarose gels by gel electrophoresis. After electrophoresis, gels were photographed and, if the genomic DNA was from a plant, the gel was placed in 0.25 N HCl depurination buffer for 15 minutes at room temperature with rotation prior to denaturation of DNA. Gels were incubated in denaturation solution for 30 minutes with gentle shaking, followed by 30 minutes incubation with neutralization solution. Capillary transfer of DNA from agarose gels to nylon membranes overnight at room temperature was achieved as described by Sambrook (1989) using 10 x SSC as the transfer buffer. The next morning, the membranes were marked before removing from the gels to indicate the location of the wells to the gels. The transferred DNA was fixed to the membranes using UV light treatment from a UV Crosslinker (Stratagene 1800), and membranes were allowed to dry at room temperature.

The second step was the actual hybridization which was carried out in a hybridization incubator (Johns Scientific Inc. Model 310) at 42°C. The membranes were first prehybridized in the hybridization tubes with 10 ml of hybridization solution in each tube for a minimum of 4 hours. The solution was removed and replaced by 5 ml of fresh hybridization solution containing the denatured DNA probe. The hybridization reaction was carried out for 12 hours in the incubator with rotation. Membranes were washed in a washing buffer, 2-3 times, for 30 minutes at a time, in a 67°C water bath. The remainder of the steps remained the same, as described for plaque hybridization (Methods 9.1).

9.3 Northern hybridization

The Northern hybridization technique (Thomas, 1980) was used to detect specific messenger RNA transcripts from total RNA using a small DNA fragment or other probe. (The overall reactions were similar to the Southern hybridization technique, which detects specific genomic DNA sequences using a DNA probe.) DNA primers, DNA or RNA fragments can all be used as probes. For accurate determination of the molecular weights of nucleic acids using gel electrophoresis techniques, the RNA molecules to be compared, must have equivalent conformations. This can be achieved by removing native secondary and tertiary structure with a variety of chemical denaturations. Among the currently available methods, glyoxal has become popular for irreversibly denaturing the RNA molecules before gel electrophoresis. Glyoxal denatures RNA and DNA irreversibly at neutral or slightly acidic pH by formation of a stable adduct with guanosine to prevent the renaturation of G-C base pairs. For further information, refer to Grossman (1980) and McMaster (1977).

First, total RNA was denatured and separated by size, using agarose gel electrophoresis (with RNase-free modifications). Glyoxal was obtained at a 40% solution (6 M), deionized by passage through a mixed-bed resin (Bio-Rad AG 501-X8), and stored at -20°C in small aliquots in tightly capped tubes. DMSO was deionized with the same way. The denaturation process was carried out by mixing 5.4 µl of RNA samples (up to 10 µg) in a sterile microfuge tube with 5.4 µl of 6 M glyoxal, 10 µl of DMSO, and 3 µl of 0.1 M sodium phosphate (pH 7.0). The mixture was incubated for 60 minutes at 50°C. While denaturing the RNA molecules at 50°C, 100 ml of agarose solution was prepared in 10 mM sodium phosphate by heating. After cooling the agarose solution to 70°C, solid sodium iodoacetate was added to a final concentration of 10 mM (to inactivate RNase). A horizontal gel was poured and the denatured RNA samples were mixed with 4 µl of loading dye and loaded on the agarose gel. Gel electrophoresis was carried out in 10 mM sodium phosphate

(pH 7.0) at 75 Volts for 2-3 hours. To maintain constant pH, buffer was circulated between the two reservoirs using a peristaltic pump. Following electrophoresis, the gel was removed from the apparatus and stained in 30 µg/ml acridine orange for 30 minutes in the dark at room temperature. After destaining in water, RNA bands were visualized by illumination with a short wavelength ultraviolet light, and photographed with Polaroid Type 55 black and white film.

RNA blots were prepared by transferring RNA molecules from the gel to Zeta-Probe membranes (Materials 8) using capillary transfer (blotting). Membranes were rinsed in 6 x SSPE before placement on the agarose gel. Capillary transfer was carried out in 20 x SSPE at room temperature overnight as described (Methods 9.2) using the RNase free procedures. The position of the samples were then marked on the membrane and the RNA fixed to the membrane using a UV crosslinker. Prior to hybridization, the membrane was shaken in a solution of 20 mM Tris-HCl, pH 8.0 at 65°C for 1 hour to remove the glyoxal. The RNA membrane was either hybridized immediately or stored dry for future hybridization.

The hybridization of radioactive-labeled probe with the RNA membrane was carried out in two steps. First, the RNA blot was prehybridized in 10 ml of hybridization solution containing 6 x SSPE, 7% SDS and 5 x Denhardt's at 37°C (for oligonucleotide probes) for 3 hours in a heat-sealable plastic bag. Hybridization followed under the same conditions for 16 hours following the addition of 5 ml of hybridization solution containing the heat denatured radioactive-labeled probe. Next the RNA blot was removed from its hybridization bag and placed in 100 ml of 5 x SSPE + 0.5% SDS buffer for a brief rinse to remove excess radioactive hybridization solution. The RNA blot was washed successively with agitation at room temperature for 15 minutes in each of the following solutions: 5 x SSPE/0.5% SDS; 2 x SSPE/0.5% SDS; 1 x SSPE/0.5% SDS. After washing, the moist membrane was wrapped in saran wrap and X-ray film exposed by autoradiography.

9.4 RNA analysis by primer extension

The primer extension method was used to map and quantitate the 5' termini of RNA and to detect mRNA. The test RNA was hybridized with an excess of an end-labeled single-stranded DNA primer. Reverse transcriptase was then used to extend this primer to produce cDNA complementary to the RNA template. The length of the resulting end-labeled cDNA, as measured by electrophoresis through a polyacrylamide gel under denaturing conditions, reflected the distance between the end-labeled nucleotide of the primers and the 5' terminus of the RNA. The yield of cDNA was approximately proportional to the concentration of the target mRNA sequences in the total preparation.

More specifically, oligonucleotides (17-40 mer) used as primers were radio-labeled by T4 polynucleotide kinase as described in Methods 1.6. The quality of the radio-labeled primers was checked using gel electrophoresis (15% minipolyacrylamide gel). To initiate the reverse transcription reaction, 0.5-30 µg of total RNA in 3 µl was mixed with 1 µl of kinased primer containing about 5 pmoles radio-labeled primer, denatured in a 90°C water bath for 1 minute, then quickly transferred to a 42°C heat block. Buffer I (4 µl) was added to the tube and the incubation was continued at 42°C for 15 minutes to allow annealing of the primer to the mRNA template. Meanwhile, the extension mix was prepared by combining 12 µl of Buffer II with 10-20 units of AMV reverse transcriptase. When the incubation was completed, the extension mix was added to the tube, mixed well, and incubated for 30-40 minutes at 42°C for reverse transcriptase-mediated cDNA synthesis. The reaction was stopped by adding 1/10 volume of DNA loading buffer to the tube. DNA samples were denatured by boiling and 1/3 of the reaction was loaded on a 5% polyacrylamide gel. The electrophoresis was performed at 50 mA for 2 hours. Finally, the gel was dried on a gel dryer for 1 hour at 80°C, and placed under X-ray film for exposure at -70°C for 1-2 days.

9.5 Ribonuclease Protection Assay

The *luxF* gene was cleaved at a single site with a restriction enzyme. Antisense *luxF* mRNA was prepared from the linearized plasmid using T7 RNA polymerase and ribonucleoside triphosphates ATP, CTP, GTP and TTP. Of the four deoxyribonucleotide triphosphates, only CTP was labeled with ^{32}P . The antisense RNA was purified by gel electrophoresis and used as the probe i.e., riboprobe, in a ribonuclease protection assay. After hybridization of the probe to the total RNA isolated from transgenic plants, reactions were treated with ribonuclease to remove single stranded probe and single stranded mRNA. The remaining double-stranded fragments were recovered by ethanol precipitation and analyzed by electrophoresis in a sequencing gel. The presence of an appropriately-sized protected RNA fragment indicates the presence but not the exact level of mRNA transcripts (as compared to Northern blot analysis or primer extension).

10. DNA Sequence Analysis

Once single-stranded template DNA was prepared, different methods were used for determining the DNA sequence of the BCOADh genes. Initially, single-stranded DNA sequencing using Klenow enzyme was employed (Vieira, 1987). Most sequencing data, however, was obtained using the single-stranded sequenase method.

10.1 Preparation of single-stranded DNA

The first step was to prepare an inoculum of M13K07 helper phage. A 5 μl aliquot of M13K07 phage stock solution was mixed with 0.5 ml of *E. coli* MV1193 culture ($\text{OD}_{600} > 0.8$) in 4 ml of melted soft agar (YT medium containing 0.7% agar) and poured immediately onto YT agar plates. Plates were incubated at 37°C for 6-12 hours to generate phage plaques. Individual plaques were transferred into tubes containing 3 ml of YT medium

with 70 µg/ml of kanamycin and shaken overnight in a 37°C incubator. The kanamycin resistance gene was carried by phage M13K07. The next day, bacterial cells and agar components were removed by centrifugation and supernatants were transferred by pipettes to new tubes and stored at 4°C (the M13K07 inoculum).

The second step was to infect *E. coli* MV1193 containing either phagemid pUC118 or pUC119 carrying the DNA fragment of interest with the M13K07 helper phage. Bacteria were inoculated in 3 ml of 2 x YT medium containing 0.001% thiamin and 150 µg/ml ampicillin and shaken at 37°C. While growing exponentially (log phase), the culture was infected with 10 µl of the M13K07 inoculum and incubated at 37°C for 1 hour 15 minutes. After this time the cells were diluted into 100 ml of 2 x YT (thi, Ap¹⁵⁰, Km⁷⁰) and allowed to grow for 14-18 hours. The cells were then collected by centrifugation (4,000 g for 10 minutes) and resuspended in 6 ml of TE buffer. Cells were lysed and proteins extracted by the addition of 4.5 ml phenol, followed by 30 second of vortex mixing, and the addition of 1.5 ml chloroform, followed by vortexing. After 10 min centrifugation 8,000 rpm, aqueous and organic layers were separated. The aqueous top layer, containing the amplified ssDNA from pUC118 or pUC119, was removed to a clean tube and extracted again with equal volume of chloroform. The resulting aqueous layer was mixed with 3 volumes of ether, vortexed and centrifuged again under the same conditions. After centrifugation, the ether layer was removed, and the single stranded phagemid DNA in the aqueous layer was precipitated by adding 1/20 volume of 3 M NaOAc (pH 7.0) and 2.5 volumes of 100% ethanol at -70°C for 30 minutes. Finally, the ssDNA was pelleted by centrifugation at 15,000 rpm for 20 minutes, dried and resuspended in 1 ml of TE buffer. The yield of ssDNA obtained by this protocol ranged 30-50 µg from 3 ml of culture.

10.2 Klenow method

Solutions required for this procedure are described in Materials 10.8. 0.5 µg ssDNA was mixed with 2 pmol sequencing primer and 5 µl of 4 x NT buffer in a total volume of 12 µl. The mixture was incubated for 5 minutes at 70°C for denaturation of the DNA, then transferred to 37°C for 10 minutes for annealing of the primer to the template. The following reagents were added to the tube in the order indicated then mixed: 1 µl of 0.25 M DTT, 1 µl of [α -³²P]dATP, 1 µl Klenow enzyme (1 unit/µl). 4 µl of this mix was dispensed into each of the four tubes labeled A, C, G, and T. These tubes contained 1 µl of ddA, ddC, ddG, or ddT mix, respectively. DNA synthesis was allowed to proceed for 15 minutes at 37°C. To terminate DNA elongation, 1 µl of chase mix was added and the reaction incubated at 37°C for another 15 minutes. For polyacrylamide gel electrophoresis, each reaction was mixed with 10 µl of sequencing loading buffer and heated at 90°C for 2 minutes. Only 2 µl of each reaction was loaded per lane on a 6% sequencing gel. Electrophoresis was carried out in 0.6 x TBE running buffer at 75 watts, 4,000 volts for 3.5 hours (short gels) or for 7.5 hours (long gels). The gel was exposed under X-ray films at -20°C overnight.

10.3 Single-stranded DNA sequenase method

Solutions required for this procedure are listed in Materials 10.8. 0.5 µg ssDNA was added to a tube containing the sequencing primer (0.03 OD/ml) and 2 µl of 5 x sequencing buffer. The reaction volume was brought to 10 µl, and denatured at 70°C for 5 minutes, followed by annealing at 37°C for 15-30 minutes. To the annealed mixture, 1 µl of 0.1 M DTT, 2 µl of 1 x labeling mix, 1 µl [α -³²P]dATP, and 2 µl of sequenase (diluted to 1-1.5 units/µl) were added in that order, and tubes incubated at room temperature for 5 minutes. DNA synthesis was stopped by dispensing 4 µl of the reaction mixture into A, G, C, and T tubes containing 2.5 µl of ddA, ddC, ddT, or ddG mixes, respectively. After 5 minutes

incubation at 37°C, 10 µl of sequencing loading buffer was added to each tube and 2 µl aliquots were loaded on sequencing gels. Gel electrophoresis and autoradiography were carried out as described above.

10.4 Double-stranded DNA sequencing using sequenase kit, version 2.0 (USB)

Most sequencing reagents were provided by the kit with the exception of [α -³⁵S]dATP, electrophoresis buffer and sequencing gel reagents. Double-stranded DNA (1 µg) in 18 µl was denatured in 2 µl of 2 N NaOH, 10 mM EDTA at room temperature for 5 minutes. DNA precipitation occurred following the addition of 8 µl of 5 M NH₄OAc (pH 7.3) and 100 µl ethanol. After centrifugation to collect the DNA, the DNA pellet was dissolved in a total volume of 10 µl (2 µl sequencing buffer, 1 µl of primer (0.5 pmol/µl), and 8 µl double-distilled water). The mixture was heated for 2 minutes at 90°C for denaturation, then transferred to a 37°C water bath for annealing. The following reagents were added: 1 µl 0.1 M DTT, 2 µl of 5 x diluted labeling mix, 1 µl [α -³⁵S]dATP and 2 µl of 8 x diluted sequenase. The reactions were mixed well and incubated at room temperature for 5 minutes. The termination reactions were initiated by transferring 3.5 µl of labeling reaction to each of four termination tubes which contained 2.5 µl of termination mixture A, C, G, or T. After incubation at 37°C for 5 minutes, the reactions were stopped by the addition of 4 µl stop solution. Aliquots of 2 µl each were run on each lane of a 6% wedge (0.2-1 mm) sequencing gel. Electrophoresis was carried out in 1 x TBS buffer at 3,000 volts for 3 hours. The polyacrylamide gel was dried on the gel drier for 1 hour at 80°C before exposure under X-ray film (without intensifying screens) at room temperature for 12-14 hours.

10.5 Computer software analysis of DNA sequence and potential amino acid sequences

The DNA Star software program was used to analyze restriction mapping data and

identify open reading frames (ORFs) in BCOADh complex clones. The resulting potential amino acid sequences were analyzed using Mail-Fasta (EMBL File Server) to search the current Swiss-Prot and PIR protein sequence databases for amino acid similarities with known proteins (Lipman and Pearson, 1985).

11. Preparation of recombinant proteins

11.1 Induction of *E. coli* recombinant gene expression

Target DNA fragments encoding proteins were ligated into bacterial expression vector pBluscript II KS+/- so that their transcription was under the control of the T7 promoter. The vectors were transformed into *E. coli* DLT101 cells which produces T7 RNA polymerase after induction with IPTG. The plasmid-containing strain was grown in LB medium with antibiotic selection until $OD_{600} = 1.0$. Induction was initiated by the addition of 0.2 mg/ml IPTG solution to a final concentration of 0.4mM and allowed to proceed for 1-2 hours. Cells were harvested by centrifugation and the cell pellet stored at -70°C until needed for protein isolation.

11.2 Recombinant protein purification from *E. coli*

Aliquots (100 μl) of induced DLT101 cultures were resuspended in 50 μl of protein sample buffer and vortexed vigorously. The samples were boiled for 3 minutes and loaded on protein gels for analysis. The gel electrophoresis was carried out in Tris-glycine buffer at 35 mA for 16 hours. After completion of the electrophoresis, gels were stained in 0.2% Coomassie brilliant blue for 10 minutes. Protein bands were identified by placing the stained gels on a light box. The protein band of interest was cut out from the gel, crashed into small pieces and loaded into a glass tube (8 x 100 mm). Dialysis tubing was fixed at the bottom end of the glass tube and proteins were eluted from the gel by electrophoresis.

11.3 Induction of mas promoters in plant tissues

The expression of foreign genes placed under the control of the *mas* promoters in transformed plants was induced by wounding and/or incubation with the plant hormone auxin (Langridge, et al., 1987). Wounding induction of *mas* promoter was achieved by slicing the leaf tissues, stem tissues, or fruit tissues of transformed plants with a razor blade, and placing them on a water-moist 3MM filter paper in petri dishes. The induction was carried out at room temperature or 4°C for a few hours to a few days. Auxin and wounding induction of the *mas* dual promoters was carried out by slicing tissues of leaf, stem or fruit and incubating on MS solid medium containing 0.5 mg/L 2,4-D in light rooms for one to ten days. The induced proteins were analyzed by immunoblot assay or biological assays.

11.4 Protein Quantitation

A standard curve was produced from known protein concentrations of a protein standard (bovine serum albumin, BSA) and used to estimate the protein concentrations in protein samples of unknown concentration using the method of Bradford, 1976. The commercial dye reagent and protein standard were provided by Bio-Rad. The dye reagent is a 5 x solution (provided by manufactures). The protein standard was dissolved in water at a final concentration of 1 mg/ml. Both were stored in a 4°C refrigerator. The standard curve was prepared in ten plastic cuvettes (1.0 cm pathwidth). 1 to 10 µl aliquots of standard BSA solution were mixed with water to a final volume of 800 µl. Next 200 µl of 5 x dye reagent was added to the cuvettes, the contents were mixed, and the absorbance of the reactions measured with a spectrophotometer (Beckman DU-70) at OD₅₉₅ within 15 minutes. Homogenized plant tissue samples were measured with the same procedures by addition of 10 µl of homogenized sample to 790 µl of water. The absorbance of the plant tissue sample was used to estimate protein concentration from the BSA standard curve (of absorbance

versus protein concentration.)

12. Western (Immuno) Blot Analysis

Western blotting or immunoblotting (Towbin, et al., 1979; Burnette, 1981) was used for the identification and quantitation of specific proteins in mixtures of proteins. Like Southern blotting, electrophoretically separated protein components were transferred from a gel to a solid support (membrane) and probed with reagents that would specifically recognize particular amino acid sequences. The probes for Western blotting usually were antibodies that reacted specifically with antigenic epitopes displayed by the target protein. The technique was modified for this project.

12.1 SDS-polyacrylamide gel electrophoretic separation of total proteins from plant tissues

Leaf or fruit tissues from transgenic tomato plants were sterilized and induced on MS + 2,4-D (0.5 mg/L) medium for maximum production of proteins when genes were driven by *mas* dual promoters. Tissues were collected after 3 days induction, and tissue weights were determined. Tissues were then homogenized by grinding in a mortar containing sea sand; three volumes (v/w) of homogenization buffer (Materials 10.5) were used for leaf tissues and 0.5 volume (v/w) of homogenization buffer for fruit tissues. The suspensions were collected, centrifuged and supernatants were mixed with 1/4 volume of sample buffer. The samples were boiled for 3 minutes, and 100 µl aliquots of each were loaded on a 3 mm 7.5-20% gradient polyacrylamide gel. Gel preparation and gel electrophoresis were performed as described previously (Methods 2.2).

12.2 Transfer of proteins from polyacrylamide gels to membranes

Proteins were transferred from polyacrylamide gels to PVDF membranes without

staining. The gel was floated in transfer buffer (Materials 10.5) for 30 minutes with rotation. Meanwhile, PVDF membrane was soaked in methanol for a few minutes, then rinsed with water, and finally stored in a transfer buffer. A sandwich structure was built (from bottom to top) with (1) 4 layers of 3MM filter paper, (2) the gel, (3) the membrane, and (4) 4 layers of 3MM filter paper (☉~☉). The 4 layers of 3MM filter papers were presoaked with transfer buffer. The sandwich structure was placed in an electro-blot apparatus (Semi-Phor, Hoefer Scientific Instruments) and protein samples electro-transferred onto the membrane at 200 mA for 3 hours.

12.3 Immunoblotting

Before immunoblotting was started, PVDF membrane containing protein samples was immersed in 100% methanol for 5 minutes, then rinsed with water for 5 minutes, followed by incubating in TBST buffer (Materials 10.5) for 5 minutes. The immunoblotting process was initiated by incubation of the membrane in freshly made blocking solution containing TBST + 1% BSA + 20% Fetal Calf Serum (Sigma) for 1 hour with shaking on a rotary shaker. The blocking solution was then replaced with TBST containing the antibody against the target protein at a dilution of 1:2,000 to 1:10,000 and incubated at room temperature for 1 hour with shaking. After incubation, the membrane was washed 3 times with TBST, for 5-10 minutes per wash. The second antibody (goat anti-rabbit alkaline phosphatase conjugate, Sigma A-3687) was added in TBST with a dilution of 1:5,000, and incubated for 30 minutes as before. The membrane was washed as before and treated for 2 minutes in alkaline phosphate buffer before transfer to a clean container. The hybridized protein band was visualized by the following color reaction. Membrane in BCIP/NBT solution (Sigma B-5655, 1 tablet in 10 ml water) was incubated in the dark for 2-60 minutes. BCIP/NBT is an insoluble alkaline phosphatase substrate, namely 5-bromo-4-chloro-3-

indolyl phosphate. The color reaction was terminated by removal of color reagents and rinsing the membrane in water.

13. Autoradiography

The ^{32}P -labelled membranes from Northern blot or Southern blot hybridization studies were placed in X-ray film cassettes with Kodak film and intensifying screens and at -70°C for the appropriate time to obtain the desired exposure intensity. ^{35}S -labelled sequencing gels were dried on a SLAB gel dryer at 80°C for 1-2 hours, then placed in X-ray film cassettes with Kodak film without intensifying screens for an overnight exposure at room temperature. Unless specified, ^{32}P -labelled polyacrylamide gels, not dried, placed on X-ray film in a film cassette with intensifying screens at -70°C for an appropriate time to obtain the desired exposure intensity. Film was developed using a Kodak X-OMAT automatic film processor.

14. Bacterial Luciferase Assays

14.1 Transgenic plants in vitro

Samples (no more than 100 mg) taken from plant tissues were put in 1.5 ml eppendorf tubes and homogenized in 500 μl luciferase assay buffer (Materials 10.7) with a plastic pestle. Homogenized 400 μl samples were transferred to 20 ml scintillation vials. The activity of luciferase was measured as the total light produced during the first 10 seconds of enzymatic reaction as detected by a luminometer (Turner TD-20e). The reaction was started by injection of an assay mixture (0.5 ml of 100 μM reduced FMN (Materials 10.7) and 20 μl of diluted decanal substrate) through the septum of the luminometer sample chamber. The FMN solution was prepared in 200 mM Tricine buffer (pH 7.0) and reduced by exposure to light from a cool-white fluorescent bulb for 10 minutes. The substrate 0.1%

(v/v) decanal was prepared in water and used immediately after sonication.

14.2 Transgenic plants *in vivo*

Low light video image analysis can be used to analyze luciferase gene expression *in vivo* (Langridge, et al., 1994). This is possible because the substrate for the assay, the long-chain aldehyde decanal, can enter living cells as a vapor. Transgenic plant tissues were placed in the center of a 14 cm diameter petri dish. 2-3 drops of decanal was added to hydrated paper wicks at the edge of the dish. The plate was closed and photon counting started after 2 minutes (to allow background fluorescence to dissipate). Photons were collected for 1 to 30 minutes and displayed on the monitor. The video image of the tissue was exhibited and overlaid with a low light image. The bioluminescence emission was quantified directly by the measurement.

15. Antisera Production

15.1 Animals and timeline

New Zealand white female rabbits, 3 months old and approximately 3-4 kilogram in weight, were used for the preparation of antibodies. The back and proximal limbs were shaved before any antigen was injected. The immunization process was divided into four steps: antigen injection, first boost, second boost, and the third boost. Sera was collected 15 days after each boost.

15.2 Preparation of antigen

E. coli produced recombinant protein (0.5-1.0 mg) was partially purified from total protein using polyacrylamide gel electrophoresis. The protein gel was stained superficially and the bands of interest were cut out from the gel. The protein-containing gel was crushed

into small pieces by passage through a syringe, with subsequent passage through finer and finer hypodermic needles (18G, 20G, 23G, 25G and finally 26G) to liquify the polyacrylamide.

15.3 Immunization of rabbits

About 0.8 ml of the protein-gel mixture (containing 0.5-1.0 mg protein antigen) was injected into the rabbits using 26G hypodermic needles. To maintain a prolonged release of antigen, the initial immunization was delivered in 10 (80-100 μ l) intradermal injections along the animal's back.

A series of three booster injections of antigen were then given by intramuscular injection of antigen (0.5-1.0 mg) in each hind quadricep muscle. The first booster was given three weeks after the initial immunization. The second booster was given 55 days after the first booster. The third booster was given 55 days after the second booster. The purpose of the booster injections was to enhance the initial weak surface antibody production of the B lymphocytes.

15.4 Immune serum collection

Fifteen days after each antigen boost, blood (30-50 ml) was withdrawn from each rabbit by a marginal ear vein bleeding to assess its production of the desired antibody. The animal was usually euthanized and bled out after the third or final antigen boost. Blood samples were centrifuged at 3,000 rpm for 30 minutes; the clear serum was collected and transferred to a beaker. Next, 25 ml of saturated ammonium sulfate solution (pH 7.8 by 2 N NaOH) was added (dropwise) to 50 ml of serum sample with constant stirring. The mixture was stirred for an additional 2 to 3 hours at room temperature then centrifuged at room temperature for 30 minutes at 1,400 x g. The resulting pellet was dissolved in 5 ml of PBS

buffer (Materials 10.5). The ammonium sulfate from the precipitate was removed by dialysis against PBS buffer for 2 days at 4°C. Dialysate was changed in the morning and the evening and tested for BaSO₄ precipitation. After dialysis was completed e.g., no BaSO₄ precipitation, the solution was removed from the tubing and aliquoted in small volumes in eppendorf tubes and stored at -20°C. The quality of antibodies was checked by immunoblotting with a series of diluted protein antigens.

16. Plant Sterilization and Germination

16.1 Sterilization of seeds, leaves, fruits, and plantlets

Tobacco or tomato seeds were packaged in pieces of miracloth (Calbiothem, Behouring Diagnostics). They were first stirred with magnetic bars in 70% ethanol for 2 minutes, rinsed once with sterile water, then transferred to 20% Clorox (1% bleach) with 2 drops of Tween 80, and sterilized for 30-35 minutes. Finally, they were rinsed 3-4 times in sterile water and left in a framehood to dry.

Tomato fruits were sterilized in 1% bleach with 2 drops of Tween 20 for 10 minutes, then rinsed with sterile distilled water 3-4 times. Tobacco or tomato leaves and plantlets were incubated in 75% ethanol for 30 minutes with stirring. They were later transferred to 1% bleach, for 10 minutes and washed with water 5 times.

16.2 Seed germination

Dried, sterilized tobacco or tomato seeds were placed on solid MS germination medium using sterile forceps: (1) about 20-30 seeds on each petri plate for tobacco and (2) about 50 seeds in magenta boxes for tomato. The petri plates or magenta boxes were sealed with parafilm and wrapped with aluminum foil. They were kept at 24-26°C for 3 days, then the aluminum foil was removed and germinated seeds were grown in light rooms under a 12

hour photoperiod for a total of 7-10 days.

17. Specimen Storage and Culture Conditions

17.1 *E. coli*

All bacterial strains were grown in 20 ml of the appropriate media to late log phase. Cells collected by centrifugation were resuspended in 1 ml of sterile 50% glycerol and transferred to a 2 ml sterile polypropylene tube for storage at -70°C.

E. coli strains were grown in LB. The growth temperature of *E. coli* strains is 37°C. For plasmid amplification, the appropriate antibiotics were added to plasmid-containing *E. coli* strains at the concentrations shown in Table 5. For the selection of recombinant plasmids in strain DH5 α , 5-bromo-4-chloro-3-indolyl galactoside (X-gal) was added to each tube of transformed cell culture at a final concentration of 2 mg/ml. For IPTG induction of *E. coli* DLT101, isopropyl β -D-thiogalactopyranoside (IPTG) was added to the culture at a final concentration of 0.4 mM 2 hours before harvesting the cells.

During conjugation with *Agrobacterium*, *E. coli* S17-1 was grown in YEB + Mg²⁺ medium. For recombinant phage isolation, *E. coli* LE392 cells were grown in 2 x YT medium or NZYMM broth prior to infection and poured onto NZY plates after mixing with 0.7% top agar (NZYMM with 0.7% agar at 50°C).

TSS medium was used for one-step *E. coli* transformation; SOB and SOC media were used for high-efficiency transformation of *E. coli* procedures.

17.2 *A. tumefaciens*

A. tumefaciens GV-3101(pMP90RK) was grown in LB supplemented with 100 mg/liter of rifampicin (Rif), 25 mg/liter of kanamycin (Km) and 25 mg/liter of gentamycin (Gm) at 29°C. During conjugation with *E. coli* S17-1, this *Agrobacterium* strain was cultured

in YEB + Mg⁺² medium supplemented with antibiotics as described above.

17.3 Bacteriophage λ

These procedures were used during the selection and rescreening of BCOADh genes from the genomic library of *B. subtilis* 168s. The detailed background information can be found elsewhere (Sambrook et al., 1989).

E. coli host strain LE392 was inoculated onto NZY plates (Materials 9.1), and grown in a 37°C incubator overnight. A single colony was picked the next day and inoculated into 10 ml NZYMM broth (Materials 9.1), cultured with shaking at 30°C overnight or at 37°C for 6 hours. NZYMM broth contained 0.2% maltose which helped bacteria adsorb bacteriophage λ efficiently. After cells were manipulated, they were collected by centrifugation and resuspended in 3 ml of 10 mM MgSO₄.

The next step was to allow the bacteriophage λ to infect host cells and amplify in the host cells. Plaques containing phage formed in the semisolid medium, resulted from a limited diffusion of the progeny virus particles. Each plaque contained genetically identical information and can be analyzed by hybridization individually.

To produce well-separated plaques, ten fold series dilutions of the bacteriophage stock were prepared in SM solution. Each diluted phage solution was dispensed into a 10 ml sterile tube (13 mm x 100 mm) and 100 μ l of plating bacteria culture was added to each tube. The phage infection was carried out first by incubation for 20 minutes at 37°C to allow the bacteriophage particles to adsorb the bacterial cells. After that time, 3 ml of melted top agar (50°C, NZYMM + 0.7% agar) was added into one tube, vortexed gently and the entire content of the tube poured immediately onto an NZYMM plate, distributed by swirling. The same procedure was performed for the rest of the tubes. The plates were moved to a 37°C incubator after the top agar became solid and incubated for 7 hours at 37°C. The plates

containing about 100 plaques per plate were chosen for further analysis, such as picking individual bacteriophage λ plaques for DNA isolation or transferring to membranes for plaque hybridization.

Individual λ plaques were picked using sterile toothpicks and transferred into 10 ml polypropylene tubes. Each tube contained 1 ml of SM solution and 1 drop of chloroform. These tubes were then kept at room temperature for 1-2 hours to allow the bacteriophage particles to diffuse out of the agar. The mixtures were then centrifuged, and supernatants containing the bacteriophages were transferred to microfuge tubes individually and stored at 4°C.

17.4 Plant growth conditions

Plant light room conditions were set for a 12 hour photoperiod, 24/20°C day/night temperature. Fluorescent tubes (cool white, 115 W) provided a light intensity of 150 $\mu\text{E}/\text{m}^2/\text{sec}$.

Potted plants were maintained in a readily mixed growth media (Metro-Mix #290) composed of vermiculite, sphagnum peat moss, sand, perlite and pH was adjusted to 6.5 with calcium carbonate. Two plant growth chambers were used under different conditions. The #1 growth chamber was set at 4°C with 16 hour photoperiod. Light was provided by very high output (V.H.O.) combination of 115 watt fluorescent tubes and 40 watt incandescent bulbs. The illuminance was 95 $\mu\text{E}/\text{m}^2/\text{sec}$ at pot height and 140 $\mu\text{E}/\text{m}^2/\text{sec}$ at plant height. Plants were watered as required, and fertilized monthly with a complete 20-20-20 fertilizer soluble solution. The #2 growth chamber was set at 22°C for 16 hours (day time) and at 18°C for 8 hours (night time). Light was provided by a V.H.O. combination of 115 watts fluorescent tubes and 40 watts incandescent bulbs at 100 $\mu\text{E}/\text{m}^2/\text{sec}$ at plant height. Plants were watered daily, and fertilized weekly with a complete 20-20-20 soluble fertilizer

solution.

Plants grown in the greenhouse compartments were under natural day light, supplemented by high intensity discharge (H.I.D.), 400 watts high pressure sodium lamps for a total of 16 hours. When using H.I.D. lighting, the maximum illuminance was 450 $\mu\text{Em}^2/\text{sec}$. The temperatures in the greenhouse were 20°C during the day, 18°C at night. Plants were watered three times daily with the following nutrients (in ppm): $\text{NO}_3(\text{N})$:20; $\text{NH}_4(\text{N})$:10; P: 20; K: 20; Ca: 10; Mg:10; Fe: 0.85; Mn: 0.55 (E.C. = 456; pH = 6.0).

RESULTS

1. Cloning and Sequencing of Branched-Chain α -Oxo Dehydrogenase Genes from *Bacillus subtilis*

1.1 Cloning of DNA fragments

Plaque hybridization was used to clone the branched-chain α -oxo acid dehydrogenase (BCOADh) from *B. subtilis*. A 920-bp PstI fragment of *B. subtilis* 168s genomic DNA (randomly-labeled with ^{32}P) was used to screen a λ EMBL3 *B. subtilis* genomic library. Phage plaques hybridizing to the probe were purified and screened twice for hybridization to the same probe. 12 positive recombinant λ plaques containing DNA inserts were purified and two clones (#7 and #11), with the strongest hybridization signals, were selected for further characterization.

1.2 Mapping of DNA fragments

The DNA from recombinant λ clones # 7 and #11 was amplified in *E. coli* LE392 and purified (Methods 17.3). Cleavage products from restriction enzyme digestion analysis were electrophoresed in 0.8% agarose gels. The restriction enzyme mapping strategy employed six single enzyme digests (BamHI, EcoRI, HindIII, PstI, Sall and XbaI), followed by 13 double enzyme digests (EcoRI/BamHI, EcoRI/HindIII, EcoRI/PstI, EcoRI/Sall, EcoRI/XbaI, BamHI/HindIII, BamHI/PstI, BamHI/Sall, BamHI/XbaI, HindIII/PstI, HindIII/Sall, HindIII/XbaI, and PstI/Sall). The DNA fragments were separated using agarose gel electrophoresis, and transferred to nylon membranes (blots). Southern hybridization analysis (Figure 6A and B), using the ^{32}P -labelled 920-bp Pst I fragment, identified those cleavage products containing BCOADh DNA sequences, resulted in the restriction mapping shown

in Figure 7.

1.3 Determination of DNA sequence

The restriction endonuclease map of clone #11 (Figure 7) displays the 0.9 Kb PstI, 2.6 Kb HindIII, and 4.5 Kb SalI fragments which were sequenced. The BCOADh genes containing PstI, HindIII and SalI DNA fragments were isolated from recombinant λ clone #11 and subcloned into plasmids pUC118 and pUC119. DNA sequencing was carried out with Klenow using universal and reverse primers (Materials 6.2). For efficient sequencing of the 2.6 Kb HindIII fragment, a series of unidirectional deletions (truncations) of the HindIII fragment was generated using exonuclease III, S1 nuclease, Klenow, and T4 DNA ligase (Methods 1.9). The products from two different deletion reactions (of different duration) were separated on agarose gels by gel electrophoresis (Figure 8 A and B) then transferred to nylon membranes and subjected to Southern hybridization analysis probing with ^{32}P -labeled 920-bp PstI DNA fragment (Figure 8 C and D). Only those truncated DNA fragments which hybridized to the probe (thus indicating the presence of BCOADh genes) were subcloned and sequenced.

More than 20 truncated subclones were sequenced using the universal primer and the reverse primer at both ends of the DNA fragments. Primer walking from the inside of the inserted DNA fragments was also carried out. Sequencing templates (single-stranded DNAs) were produced (Methods 10.1) and tested by gel electrophoresis (Figure 9). A total of 25 synthetic oligonucleotide primers were used to carry out the sequencing 3,700 bp of DNA on both strands (Figure 10) using several different sequencing techniques (Methods 10.2 & 10.3).

The 3,700 bp nucleotide sequence obtained (Figure 11) includes three open reading frames (ORFs). The first ORF encodes a polypeptide chain of 330 amino acids (36,310 Da),

the second ORF encodes a polypeptide chain of 327 amino acids (35,893 Da), and the third ORF encodes a polypeptide chain of 424 amino acids (45,803 Da). Seven bases upstream from the ATG codon of the first open reading frame, there was a five-base sequence, GGAGG (position -14 to -8, Figure 11). Because of its complementarity with the 3' end of the 16S rRNA from *B. subtilis* (Moran et al., 1982), this sequence is most likely a ribosome-binding site (i.e., a Shine-Dalgarno sequence) for the first ORF. The 5'- GGAGG -3' sequence was also found in front of the second and third open reading frames. A palindromic sequence, which is likely to act as a transcriptional terminator, appears at the end of the third open reading frame (Figure 11). The first and the second ORFs were separated by 13 nucleotides, and the second and the third ORFs were separated by 21 nucleotides.

1.4 Similarities between ORFS and BCOADh enzyme complex amino acid sequence

The amino acid sequences of the three ORFs (Figure 11) were compared to the sequences of known proteins deposited in the Swiss-Protein computer database (Table 9). ORF1 (330 amino acids) is quite similar to the E1 α component of several branched-chain α -oxo acids, pyruvate and 2-oxo glutarate dehydrogenase complexes; it exhibits between 30-38% identity with the 296-322 amino acids of these known genes. ORF2 (327 amino acids) is similar to the E1 β component of several dehydrogenase complexes; it exhibits between 37-49% identity with the 319-338 amino acids of these known genes. ORF3 (424 amino acids), shows similarity with the E2 component of the dehydrogenase complex; it exhibits between 32-38% identity with the 250-431 amino acids of E2 genes.

Further support for the identification of ORF1 as E1 α came from the presence of both a thiamin-diphosphate-binding motif (Figure 12) and putative substrate interaction and phosphorylation sites (Figure 13). A thiamin-diphosphate-binding motif has been postulated for many thiamin-diphosphate-requiring enzymes (Hawkins, et al., 1989), and the enzyme

subunits of α -oxo acid dehydrogenase have been shown to require thiamin diphosphate as a cofactor. It is interesting to note that even the activity of this bacterial enzyme (e.g., in *B. subtilis* and *P. putida*) is not regulated by phosphorylation, as it is in animals, phosphorylation sites are still present.

Further support for the identification of ORF2 as E1 β showed four regions exhibiting a high degree of amino acid similarity with pyruvate and branched-chain α -oxo acid dehydrogenase from human, bovine, rat and *P. putida* species (including positions 25-60, 141-177, 228-263 and 268-301). In particular, regions two and three are very similar to regions thought to be involved in the decarboxylation of pyruvate and the branched-chain α -oxo acids in other organisms (Wexler, 1991; Koike, 1990).

1.5 Summary

The BCOADh enzyme complex in many organisms contains enzymes E1(α and β subunits), E2, and E3. E1 encodes the decarboxylase activity of the BCOADh enzyme complex. The first objective of this project was to clone and sequence genes encoding the BCOADc activity of the *B. subtilis* BCOADh enzyme complex. A 920-bp PstI fragment of *B. subtilis* genomic DNA, known to restore function to a fatty-acid requiring (*bfn*) strain of *B. subtilis*, was used to clone genes encoding enzymes of the *B. subtilis* BCOADh enzyme complex. Identification of ORF1 as E1 α and ORF2 as E1 β has met this first objective.

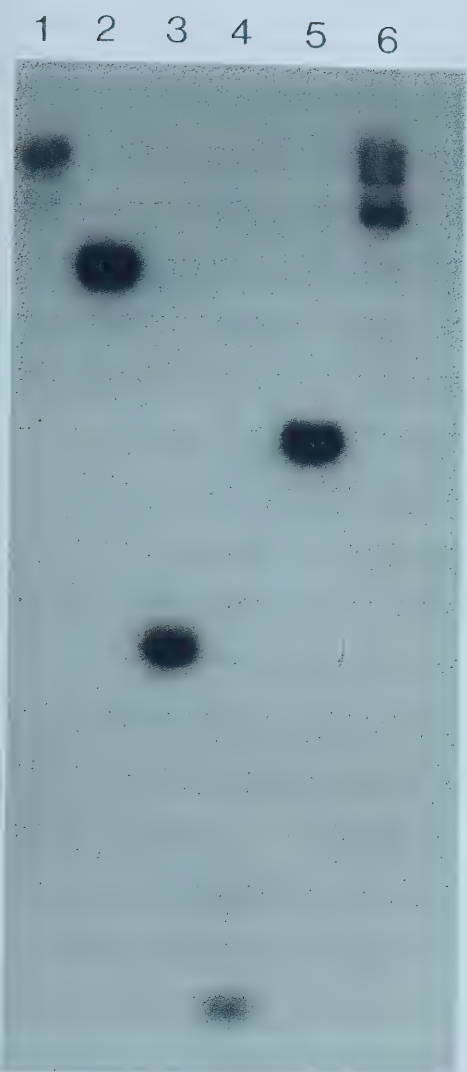
In addition, ORF3 has been identified as E2, which may encode a transacylase activity. DNA sequences similar to E3 were not found in the clones characterized in this project. One possible explanation is that the *B. subtilis* E3 gene is not linked to the E1 and E2 genes. If E3 is shared by three different dehydrogenase enzyme complexes— pyruvate, 2-oxo glutarate and branched-chain α -oxo acid—it may not lie in the same operon as genes for E1 and E2.

Figure 6. Localization of BCOADh genes in EMBL 3 λ recombinant #11 by Southern hybridization. DNA fragments from either single (panel A) or double (panel B) restriction enzyme digests were hybridized with a ^{32}P -labeled 920-bp PstI fragment followed by 8 hours of exposure on X-ray film.

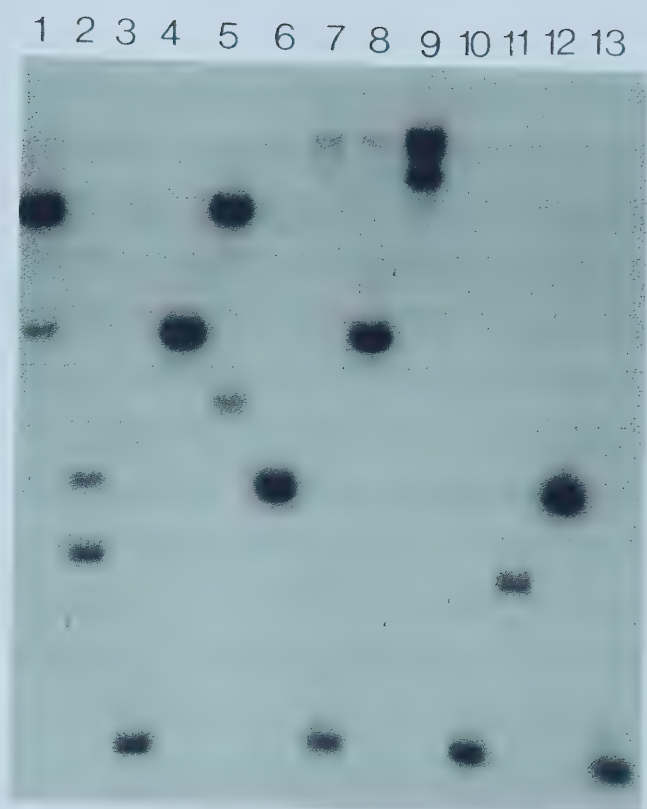
Panel A: λ DNA from #11 recombinant phage DNA digested with lane 1, BamHI; lane 2, EcoRI; lane 3, HindIII; lane 4, PstI; lane 5, Sall; lane 6, XbaI.

Panel B: λ DNA from #11 recombinant phage DNA digested with lane 1, EcoRI/ BamHI; lane 2, EcoRI/HindIII; lane 3, EcoRI/PstI; lane 4, EcoRI/Sall; lane 5, EcoRI/XbaI; lane 6, BamHI/HindIII; lane 7, BamHI/PstI; lane 8, BamHI/PstI; lane 9, BamHI/XbaI; lane 10, HindIII/PstI; lane 11, HindIII/Sall; lane 12, HindIII/XbaI; lane 13, PstI/Sall.

A



B



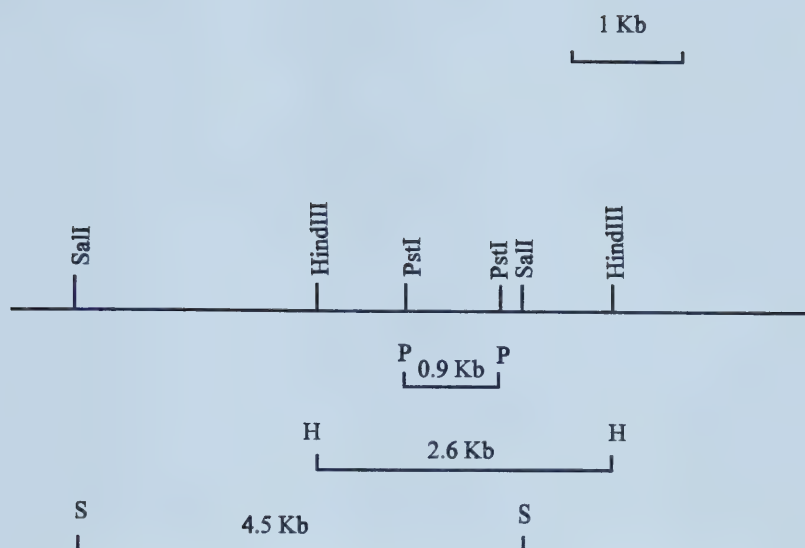


Figure 7. Restriction map of recombinant λ #11. P, PstI; H, HindIII; S, SalI; Kb, Kilobase pairs.

Figure 8. Truncation of a 2.6 Kb HindIII DNA fragment for DNA sequencing.

Panel A. Ethidium bromide stained gel of set 1 deletions. M, λ /HindIII molecular weight markers. Lanes A, no deletion; B, 30 seconds; C, 60 seconds; D, 90 seconds; E, 120 seconds; F, 150 seconds; and G, 180 seconds.

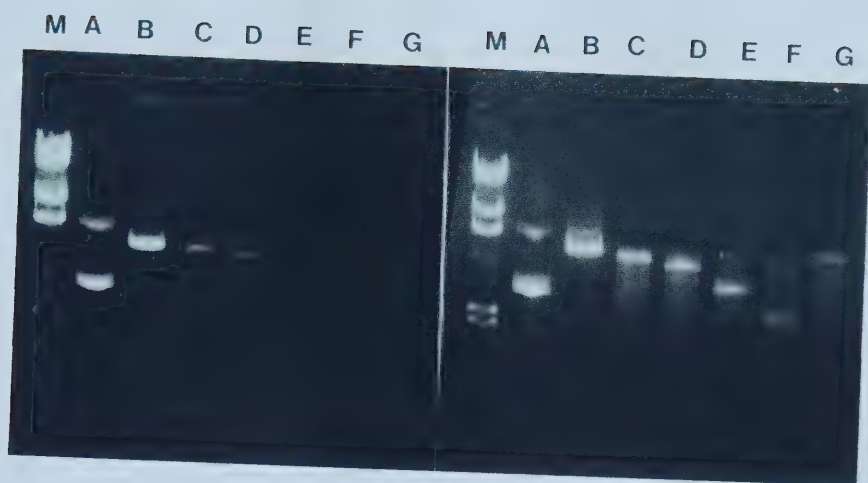
Panel B. Ethidium bromide stained gel of set 2 deletions. M, λ /HindIII molecular weight marker. Lanes A-G contain the products of deletion reactions stopped at 0, 1, 2, 3, 4, 5, and 6 minutes, respectively.

Panel C: Southern blot of gel in panel A to identify truncated fragments containing DNA sequences homologous to the 920-bp fragment.

Panel D: Southern blot of gel in panel B for the same purpose.

A

B



C

D

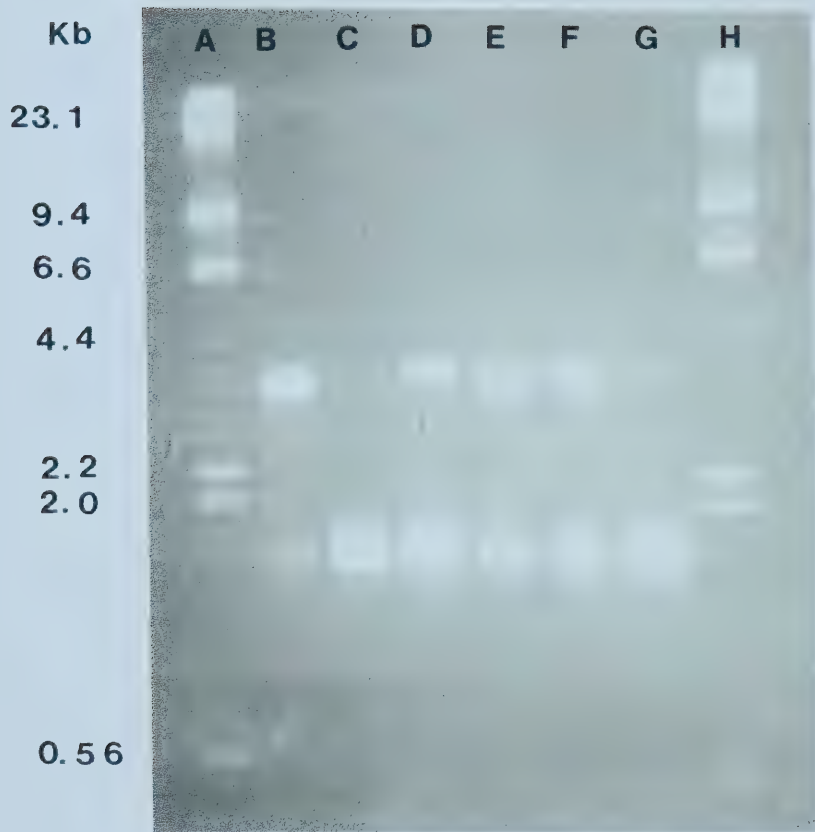
A B C D E F G

A B C D E F G



Figure 9. Analysis of single-stranded DNA produced from pUC118-HindIII subclones in *E. coli* MV1193.

Lanes A and H, λ /HindIII molecular weight markers showing DNA bands with 23.1, 9.4, 6.6, 4.4, 2.2, 2.0, and 0.56 kilobase pairs in length; lanes B-G, ssDNA samples from six individual subclone constructs. The electrophoresis was carried out in 0.7% agarose gels and DNA was stained with ethidium bromide and photographed with a Polaroid camera system.



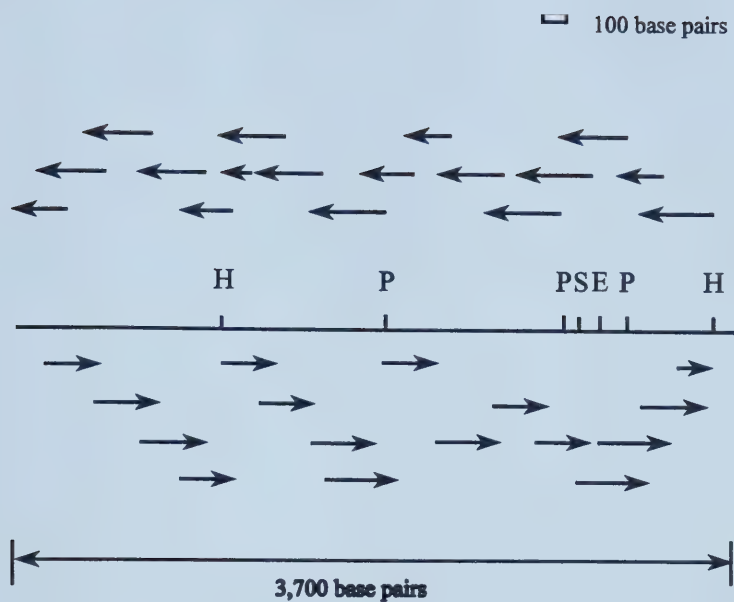


Figure 10. Strategy of two direction DNA sequencing of 3.7 Kb DNA fragment of *Bacillus subtilis* 168s genomic DNA. H, HindIII; P, PstI; S, SalI; E, EcoRI. Each arrow indicates the position, direction and the length of a DNA fragment sequenced by a single primer.

Figure 11. DNA sequence and putative amino acid sequence of a 3,700 bp region containing three ORFs of BCOADh genes (E1 α , E1 β and E2). Putative ribosome binding sites and transcriptional terminators are indicated by underlining of sequences and by arrows, respectively.

1 AGATACAGATGATATTTCTCGGCGTTCATATGATTGGCCCGCATGTGCACCGACATGATTTCTGAAGCGGGTCTTGCCAAA

2 GTGCTGGACGCAACACCGTGGGAGGTCGGGCAAACGATTCACCCGCATCCAACGCTTTCTGAAGCAATTGGAGAAGCTGC
1st ORF (E1α)

3 GCTTGCCGCAGATGGCAAAGCCATTTCATTTTAAAGCATAAAGGAGGGGCTTGAATGAGTACAAACCGACATCAAGCAC
Met Ser Thr Asn Arg His Gln Ala

4 TAGGGCTGACTGATCAGGAAGCCGTTGATATGTATAGAACCATGCTGTTAGCAAGAAAAATCGATGAAAGAATGTGGCTG
Leu Gly Leu Thr Asp Gln Glu Ala Val Asp Met Tyr Arg Thr Met Leu Leu Ala Arg Lys Ile Asp Glu Arg Met Trp Leu

5 TTAAACCGTTCTGGCAAATTCATTGTAACTCTCTTGTCAGGACAGGAAGCAGCACAGGTAGGAGCGGCTTTCGCAT
Leu Asn Arg Ser Gly Lys Ile Pro Phe Val Ile Ser Cys Gln Gly Gln Glu Ala Ala Gln Val Gly Ala Ala Phe Ala Leu

6 TGACCGTGAAATGGATTATGTATTGCGTACTACAGAGACATGGGTGTCGTGCTCGCGTTTGGCATGACAGCAAAGGACT
Asp Arg Glu Met Asp Tyr Val Leu Pro Tyr Tyr Arg Asp Met Gly Val Val Leu Ala Phe Gly Met Thr Ala Lys Asp

7 TAATGATGTCCGGGTTTGCAAAGCAGCAGATCCGAAGTCAGGAGGCCGCGCAGATGCCGGGACATTTCCGACAAAAAGAAA
Leu Met Met Ser Gly Phe Ala Lys Ala Ala Asp Pro Asn Ser Gly Gly Arg Gln Met Pro Gly His Phe Gly Gln Lys Lys

8 AACCGCATTGTGACGGGATCATCTCCGTTTACAACGCAAGTGCCGCACGCAGTCGGTATTGCGCTTGCGGGACGTATGGA
Asn Arg Ile Val Thr Gly Ser Ser Pro Val Thr Thr Gln Val Pro His Ala Val Gly Ile Ala Leu Ala Gly Arg Met Glu

9 GAAAAAGGATATCGCAGCCTTTGTTACATTCGGGGGAAGGGTCTTCAAACCAAGGCGATTTCCATGAAGGGGCAAACCTTTG
Lys Lys Asp Ile Ala Ala Phe Val Thr Phe Gly Glu Gly Ser Ser Asn Gln Gly Asp Phe His Glu Gly Ala Asn Phe

10 CCGCTGTCCATAAGCTGCCGTTATTTTCATGTGTGAAAAACAACAAATACGCAATCTCAGTGCCTTACGATAAGCAAGTC
Ala Ala Val His Lys Leu Pro Val Ile Phe Met Cys Glu Asn Asn Lys Tyr Ala Ile Ser Val Pro Tyr Asp Lys Gln Val

11 GCATGTGAGAACATTTCCGACCGTGCCATAGGCTATGGGATGCCTGGCGTAACTGTGAATGAAATGATCCGCTGGAAGT
Ala Cys Glu Asn Ile Ser Asp Arg Ala Ile Gly Tyr Gly Met Pro Gly Val Thr Val Asn Gly Asn Asp Pro Leu Glu Val

12 TTATCAAGCGGTTAAAGAAGCACGCGAAAGGGCACGCAGAGGAGAAGGCCGACATTAATTGAAACGATTTCTTACCGCC
Tyr Gln Ala Val Lys Glu Ala Arg Glu Arg Ala Arg Gly Glu Glu Fly Pro Thr Leu Ile Glu Thr Ile Ser Tyr Arg

13 TTACACCACATTCAGTGATGACGATGACAGCAGCTACAGAGGCCGTGAAGAAGTAGAGGAAGCGAAAAAAGTGATCCC
Leu Thr Pro His Ser Ser Asp Asp Asp Ser Ser Tyr Arg Gly Glu Glu Val Glu Glu Ala Lys Lys Ser Asp Pro

14 CTGCTTACTTATCAAGCTTACTTAAAGGAAACAGGCCTGCTGTCCGATGAGATAGAACAAACCATGCTGGATGAAATTAT
Leu Leu Thr Tyr Gln Ala Tyr Leu Lys Glu Thr Gly Leu Leu Ser Asp Glu Ile Glu Gln Thr Met Leu Asp Glu Ile Met

15 GGCAATCGTAAATGAAGCGACGGATGAAGCGGAGAAGCGCCCATATGCAGCTCCTGAGTCAGCGCTTGATTATGTTTATG
Ala Ile Val Asn Glu Ala Thr Asp Glu Ala Glu Asn Ala Pro Tyr Ala Ala Pro Glu Ser Ala Leu Asp Tyr Val Tyr
2nd ORF (E1β)

16 CGAAGTAGGGAGGAAGAACAATGTCAGTAATGTCTATATTGATGCAATCAATTTGGCGATGAAAGAAGAAATGGAACG
Ala Lys - Met Ser Val Met Ser Tyr Ile Asp Ala Ile Asn Leu Ala Met Lys Glu Glu Met Glu Arg

17 AGATTCTCGCGTTTTCGTCTTGGGGAAGATGTAGGAAGAAAAGCGGTGTGTTTAAAGCGACAGCGGGACTCTATGAAC
Asp Ser Arg Val Phe Val Leu Gly Glu Asp Val Gly Arg Lys Gly Gly Val Phe Lys Ala Thr Ala Gly Leu Thr Glu

18 AATTGTTGGGAAGAGCGCGTTATGGATACGCCGCTTGCTGAATCTGCAATCGCAGGAGTCGGTATCGGAGCGGCAATGTAC
Gln Phe Gly Glu Glu Arg Val Met Asp Thr Pro Leu Ala Glu Ser Ala Ile Ala Gly Val Gly Ile Gly Ala Ala Met Tyr

19 GGAATGAGACCGATTGCTGAAATGCAGTTTGCTGATTTCATTATGCCGGCAGTCAACCAAATTATTTCTGAAGCGGCTAA
Gly Met Arg Pro Ile Ala Glu Met Gln Phe Ala Asp Phe Ile Met Pro Ala Val Asn Gln Ile Ile Ser Glu Ala Ala Lys

20 AATCCGCTACCGCAGCAACAATGACTGGAGCTGTCCGATTGTGCTCAGAGCGCCATACGGCGGAGGCGTGCACGGAGCCC
Ile Arg Tyr Arg Ser Asn Asn Asp Trp Ser Cys Pro Ile Val Val Arg Ala Pro Tyr Gly Gly Gly Val His Gly Ala

21 TGTATCATTTCTCAATCAGTCGAAGCAATTTTCGCCAACCCAGCCCGACTGAAAAATTGTCATGCCATCAACACCATATGAC
Leu Tyr His Ser Gln Ser Val Glu Ala Ile Phe Ala Asn Gln Pro Gly Leu Lys Ile Val Met Pro Ser Thr Pro Tyr Asp

22 GCGAAAGGGCTCTTAAAGCCGAGTTCTGACGAAGACCCCGTCTGTTTTTGTAGACAAGCGGGCATAACCGTCTGAT
Ala Lys Gly Leu Leu Lys Ala Ala Val Arg Asp Glu Asp Pro Val Leu Phe He Glu His Lys Arg Ala Thr Arg Leu Ile

23 AAAGGGCGAGGTTCCGGCTGATGATTATGTCCTGCCAATCGGCAAGGCGGACGTAAAAAGGGAAGGCGACGACATCACAG
Lys Gly Glu Val Pro Ala Asp Asp Tyr Val Leu Pro Ile Gly Lys Ala Asp Val Lys Arg Glu Gly Asp Asp Ile Thr

24 TGATCACATACGGCCTGTGTGTCCACTTCGCCTTACAAGCTGCAGAACGTCTCGAAAAAGATGGCATTTCAGCGCATGTG
Val Ile Thr Tyr Gly Leu Cys Val His Phe Ala Leu Gln Ala Ala Glu Arg Leu Glu Lys Asp Gly Ile Ser Ala His Val

25 GTGGATTTTAAGAACAGTTTACCCGCTTGATAAAGAAGCCATCATCGAAGCTGCGTCCAAAACCTGGAAAGTTCTTTTGGT
Val Asp Leu Arg Thr Val Tyr Pro Leu Asp Lys Glu Ala Ile Ile Glu Ala Ala Ser Lys Thr Gly Lys Val Leu Leu Val

26 CACAGAAGATACAAAAGAAGGCAGCATCATGAGCGAAGTAGCCGCAATTATATCCGAGCATTGTCTGTTCGACTTAGACG
Thr Glu Asp Thr Lys Glu Gly Ser Ile Met Ser Glu Val Ala Ala Ile Ile Ser Glu His Cys Leu Phe Asp Leu Asp

27 CGCCGATCAAACGGCTTGCAAGTCTGATATTCGGCTATGCCTTATGCGCCGACAATGGAAAAATACCTTTATGGTCAAC
Ala Pro Ile Lys Arg Leu Ala Gly Pro Asp Ile Pro Ala Met Pro Tyr Ala Pro Thr Met Glu Lys Tyr Phe Met Val Asn
3rd ORF (E2)

28 CCTGATAAAGTGAAGCGGCGATGAGAGAATTAGCGGAGTTTTAAAGACGTAAGGGAGGATACAATCATGGCAATTGAAC
Pro Asp Lys Val Glu Ala Ala Met Arg Glu Leu Ala Glu Phe - Met Ala Ile Glu

29 AAATGACGATGCCGACGCTTGGAGAAAGCGTAACAGAGGGGACGATCAGCAAATGGCTTGTGCCCCCGGTGATAAAGTG
Gln Met Thr Met Pro Gln Leu Gly Glu Ser Val Thr Glu Gly Thr Ile Ser Lys Trp Leu Val Ala Pro Gly Asp Lys Lys Val

30 AACAAATACGATCCGATCGCGGAAGTCATGACAGATAAGGTAAATGCAGAGGTTCCGTCCTCTTTTACTGGTACGATAAC
Asn Lys Tyr Asp Pro Ile Ala Glu Val Met Thr Asp Lys Val Asn Ala Glu Val Pro Ser Ser Phe Thr Gly Thr Ile Thr

31 AGAGCTTGTGGGAGAAGAAGGCCAAACCTGCAAGTCGGAGAAATGATTTGCAAAATTGAAACAGAAGGCGCGAATCCGG
Glu Leu Val Gly Glu Glu Gly Gln Thr Leu Gln Val Gly Gln Met Ile Cys Lys Ile Glu Thr Glu Gly Ala Asn Pro

32 CTGAACAAAAACAAGAACAGCCAGCAGCATCAGAAGCCGCTGAGAACCTGTTGCAAAAAGTGCTGGAGCAGCCGATCAG
Ala Glu Gln Lys Gln Gln Gln Pro Ala Ala Ser Glu Ala Ala Glu Asn Pro Val Ala Lys Ser Ala Gly Ala Ala Asp Gln

33 CCCAATAAAAAGCGCTACTCGCCAGCTGTTCTCCGTTTGGCCGGAGAGCAGGGCATTGACCTCGATCAAGTGACAGGAAC
Pro Asn Lys Lys Arg Tyr Ser Pro Ala Val Leu Arg Leu Ala Gly Glu His Gly Ile Asp Leu Asp Gln Val Thr Gly Thr

34 TGGTGCCGGCGGGCGCATCACACGAAAAGATATTACGCGCTTAATTGAAACAGGCGGCGTGCAAGAACAGAATCCTGAGG
Gly Ala Gly Gly Arg Ile Thr Arg Lys Asp Ile Gln Arg Leu Ile Glu Thr Gly Gly Val Gln Flu Gln Asn Pro Glu

35 AGCTGAAAACAGCAGCTCCTGCACCGAAGTCTGCATCAAAACCTGAGCCAAAAGAAGAGACGTCATACCTGCGTCTGCA
Glu Leu Lys Thr Ala Ala Pro Ala Pro Lys Ser Ala Ser Lys Pro Glu Pro Lys Glu Glu Thr Ser Tyr Pro Ala Ser Ala

36 GCCGGTGATAAAGAAATCCCTGTACAGGTGTAAGAAAAGCAATTGCTTCCAATATGAAGCGAAGCAAAACAGAAATTCC
Ala Gly Asp Lys Glu Ile Pro Val Thr Gly Val Arg Lys Ala Ile Ala Ser Asn Met Lys Arg Ser Lys Thr Glu Ile Pro

37 GCATGCTTGGACGATGATGGAAGTCGACGTCACAAATATGGTTGCATATCGCAACAGTATAAAAGATTCTTTTAAGAAGA
His Ala Trp Thr Met Me Glu Val Asp Val Thr Asn Met Val Ala Tyr Arg Asn Ser Ile Lys Asp Ser Phe Lys Lys

38 CAGAAGGCTTTAATTTAACGTTCTTCGCCTTTTTTGTAAAAGCGGTCGCTCAGGCGTTAAAAGAATCCCCGCAATGAAT
Thr Glu Gly Phe Asn Leu Thr Phe Phe Ala Phe Phe Val Lys Ala Val Ala Gln Ala Leu Lys Glu Phe Pro Gln Met Asn

39 AGCATGTGGGCGGGGACAAAAATTATTCAGAAAAAGGATATCAATATTTCAATTGCAGTTGCCACAGAGGATTCTTTATT
Ser Met Trp Ala Gly Asp Lys Ile Ile Gln Lys Lys Asp Ile Asn Ile Ser Ile Ala Val Ala Thr Glu Asp Ser Leu Phe

40 TGTTCCGGTGATTAATAAACGCTGATGAAAAACAATTAAGGCATTGCGAAAGACATTACCGGCCTAGCTAAAAAAGTAA
Val Pro Val Ile Lys Asn Ala Asp Glu Lys Thr Ile Lys Gly Ile Ala Lys Asp Ile Thr Gly Leu Ala Lys Val

41 GAGACGGAAAACTCACTGCAGATGACATGCAGGGAGGCACGTTTACCGTCAACAACACAGGTTTCGTTCCGGTCTGTTTCAG
Arg Asp Gly Lys Leu Thr Ala Asp Asp Met Gln Gly Gly Thr Phe Thr Val Asn Asn Thr Gly Ser Phe Gly Ser Val Gln

42 TCGATGGGCATTATCAACTACCTCAGGCTGCGATTCTTCAAGTAGAATCCATCGTCAAACGCCGGTTGTCATGGACAA
Ser Met Gly Ile Ile Asn Tyr Pro Gln Ala Ala Ile Leu Gln Val Glu Ser Ile Val Lys Arg Pro Val Val Met Asp Asn

43 TGGCATGATTGCTGTGCAGAGACATGGTTAATCTGTGCCTGTCATTAGATCACAGAGTGCTTGACGGTCTCGTGTGCGGAC
Gly Met Ile Ala Val Arg Asp Met Val Asn Leu Cys Leu Ser Leu Asp His Arg Val Leu Asp Gly Leu Val Cys Gly

44 GATTCCCTCGGACGAGTGAACAAATTTTAGAATCGATTGACGAGAAGACATCTGTTTACTAAATAAGCAAAAAGAGCATT
Arg Phe Leu Gly Arg Val Lys Gln Ile Leu Glu Ser Ile Asp Glu Lys Thr Ser Val Tyr - TTTTGAAGTCTGTTTCTATGCTTTATTATTCAGCGATCCGTAATTTTCATTTCGACTCGATAT

45 TTTTGAAGTCTGTTTCTATGCTTTATTATTCAGCGATCCGTAATTTTCATTTCGACTCGATAT

46 TCTTCTGTGTTTTTCGGGGAGTAATGAATCGGTATGATTAACGTCGTATACATCACTGACAACGTGTTAATTGGCGGTCCGC

47 GATATATTTGATAAGCTT

Table 9. Similarity of amino acid sequences among components of *B. subtilis* branched-chain α -oxo acid dehydrogenase complex and those of closely related enzymes.

Component	Dehydrogenase Complex	Source	Identical Amino Acids %	Amino Acids Overlap
E1 α	branched-chain α -oxo	human	37.3	319
		bovine	36.7	319
		rat	37.9	319
	pyruvate	<i>B. subtilis</i>	37.6	322
		<i>S. cerevisiae</i>	29.9	314
		human	30.4	322
	2-oxoglutarate	<i>P. putida</i>	34.1	296
E1 β	branched-chain α -oxo	bovine	39.6	326
	pyruvate	<i>B. subtilis</i>	47.5	324
		<i>B. stearothermophilus</i>	48.9	321
		human	37.3	319
	2-oxoglutarate	<i>P. putida</i>	38.8	338
E2	branched-chain α -oxo	bovine	32.3	418
	pyruvate	<i>B. subtilis</i>	35.7	339
		<i>B. stearothermophilus</i>	35.8	427
		<i>E. coli</i>	31.6	431
	2-oxoglutarate	<i>B. subtilis</i>	34.8	422
		<i>E. coli</i>	38.0	250

Source Component		Sequence	Identical amino acids
		143 ***** *** * ***** * *** * ** ***** * 190	
<i>B. subtilis</i>	E1 α	KKD IAAFVY F GEGSSNQ G DFHEG A NFAAVHKL P VIFMCENN KYAIS V P	48
human	E1 α	NANRVVIC Y F GEGAA S EGDAHDGF N FAAT LEC P I I FF CRNN GYA I S T P	22
Bovine	E1 α	NANRVVIC Y F GEGAA S EGDAHAGFN F AATL E C P I I FF CRNN G YA I S T P	22
Rat	E1 α	NANQIV IC Y F GEGAA S EGDAHAGFN F AATL E C P I I FF CRNN G YA I S T P	22
<i>P. putida</i>	E1 α	GDTKIASAW IGDGATAES D F HTALTFAHVYR A P VILNVVNNQW A I S T F	17

Figure 12. The thiamin-diphosphate-binding motif of the E1 α component of BCOA dehydrogenase complex from different sources.

Source Component		Sequence	Identical amino acids
		<u>Putitative subunit interaction site</u>	
		206	251
		*** * *** ** * * * * * * * * *	
<i>B. subtilis</i>	E1α	GYGMPGVTVNGNDPL EVYQAVKEAR ERARRGEGPTLIET IS YRLTP	45
human	E1α	GYGIMS I RV DGNDVFAVYNAT KEARRRAVAENQ PFLIEAMTYRIGH	23
Bovine	E1α	GYGILS I R V DGNDVFAVYNAT KEARRRAVAEN Q PFLIEAMTYRIGH	23
Rat	E1α	GYGIMS IRVD GNDVFAVYN ATKEARRRAVA EN QPFLIEAMTYRIGH	23
<i>P. putida</i>	E1α	GCGIAS I RVD GNDVFAVYN ASRWAARRARRG LGPSLIEWVTYRAGP	21

Source Component		Sequence	Identical amino acids
		<u>Phosphorylation sites</u>	
		253 , 268	
		* * * * * * *	
<i>B. subtilis</i>	E1α	HS S D D D D S S Y R G R E E V E	17
human	E1α	HS T S D D D S S A Y R S V D E V N	9
Bovine	E1α	HS T S D D D S S A Y R S V D E V N	9
Rat	E1α	HS T S D D D S S A Y R S V D E V N	9
<i>P. putida</i>	E1α	HS T S D D P S K Y R P A D D W S	7

Figure 13. Putative subunit interaction and phosphorylation sites in two regions of the E1α gene of BCOADh from different sources.

2. Transformation of Tobacco and Tomato Plants with *B. subtilis* BCOADc Genes

2.1 Construction of plant expression vectors encoding BCOADc genes

Six major steps were required for subcloning A (E1 α) and B (E1 β) genes of *B. subtilis* BCOADc into plant expression vectors. These steps (in chronological order) were (1) design of oligonucleotide primers for the excision of A and B genes from the genomic clone; (2) amplification of the A and B genes *in vitro* by polymerase chain reactions (PCR); (3) subcloning of amplified A and B genes into the sequencing vector pBluescript II KS +/-, followed by transformation of *E. coli* DH5 α ; (4) DNA sequencing of A and B genes to confirm correct sequence; (5) subcloning of the A and B genes into plasmid pPCV701; and (6) subcloning of the B gene into plasmid pPCV701-*luxFP1M1*.

In step 1, four oligonucleotide primers (A1, A2, B1, and B2, see Materials 6.3) were designed with specific restriction endonuclease recognition/cleavage sites and 20-22 nucleotides of A or B gene coding sequences (Figure 14). The cloning sites present in plant expression vector pPCV701 determined which sites were introduced using the primers. A unique SalI cloning site was available for inserting foreign genes downstream (and under the control of) the mannopine synthetase (*mas*) P1 promoter. A unique BamHI site was available for inserting foreign genes downstream (and under the control of) the *mas* P2 promoter. Genomic subclone 119-S-75, containing the 4.5 Kb SalI fragment (Results 1.3), was selected as the source of A and B genes. Primers A1 and A2, containing a SalI cleavage site were used to excise the A gene encoding E1 α from the genomic DNA using PCR. Primers B1 and B2, containing the BamHI cleavage sites were used to excise the B gene of E1 β from the genomic DNA in a similar manner.

In step 2, polymerase chain reactions were carried out (Methods 1.10) to amplify the

A and B gene fragments from the genomic SalI clone (Figure 15).

In step 3, the two PCR products (A and B) were independently subcloned into pBluescript II KS +/- (a cloning and sequencing vector). Several subclones from each construct were selected and verified by restriction mapping.

In step 4, the correct DNA sequence of A gene (~1 Kb) and B gene (~1 Kb) was confirmed by dsDNA sequencing using sequenase. It was important to establish that the DNA of A and B gene fragments amplified by PCR and subcloned into plasmid pBluescript II KS+/- were identical to the original sequences in the genomic DNA clone of *B. subtilis* 168s.

In step 5, both SalI and BamHI fragments containing the A and B genes, respectively, were isolated from pBlue-A1 and pBlue-B1 and inserted at unique SalI and BamHI cloning sites of plant expression vector pPCV701. The resulting vector, pPCV701-AB, should now produce E1 α and E1 β subunit proteins under the control of the dual *mas* promoter, P1 and P2 (Figure 16).

In step 6, only the BamHI fragment containing the B gene was isolated from pBlue-B1 and inserted into the unique BamHI cloning site of plant expression vector pPCV701-*luxFP1M1* to produce vector pPCV701-*luxFP1-B* (Figure 17). Since auxin or wounding simultaneously induce both *mas* promoters, the appearance of luciferase activity in plant tissues is a direct evidence of *luxF* gene transcription and B gene transcription. The *luxF* fusion protein monomer is fully active in plant cells and its activity can be monitored *in vitro* or *in vivo* throughout plant development.

2.2 *Agrobacterium-mediated transformation of tobacco leaf-discs*

Conjugal transfer of plant expression vectors pPCV701-AB and pPCV701-*luxFP1-B* from *E. coli* to *Agrobacterium* (Methods 3.3) required that the *E. coli* host first modify the

plasmid DNA in order to prevent its restriction in the *Agrobacterium* host. *E. coli* S17-1 was selected as the donor host because it has the appropriate genotype for both modification and conjugal transfer. *Agrobacterium tumefaciens* GV3101 (pMP90RK) was selected as the recipient host. Each plant expression vector was then transferred from the transformed *Agrobacterium* host into tobacco plants (SR1) using leaf-disc infection methods (Methods 3.4; Marton, et al., 1979; De Block, et al., 1985; and Horsch et al., 1985). In addition, tobacco plants were transformed with a third expression vector pPCV701-*luxAB* as a positive control for the transformation and regeneration methods. Plasmid pPCV701-*luxAB* (Koncz, et al., 1987) was selected because both bacterial luciferase A and B genes were transcribed by the dual *mas* promoter and luciferase activity in plant tissues was fully functioning (Figure 18).

Transgenic tobacco plants were selected by kanamycin resistance (100mg/L kanamycin in media). Plantlets containing a plant expression vector with the neomycin phosphotransferase (NPTII) gene would survive and regenerate to fully grown, fertile plants. At a low frequency, plantlets which have not been transformed can also exhibit a kanamycin resistant phenotype (which will be described below). Eight weeks was required for a small plantlet to be regenerated after leaf disc transformation. An additional four-six weeks was required to obtain vegetative plants (1 meter in height). Twenty-five plants were regenerated from the pPCV701-*luxFP1-B* transformation and forty-three plants were regenerated from pPCV701-AB transformation. These plants were grown to maturity and maintained in the greenhouse.

2.3 *Agrobacterium*-mediated transformation of *L. esculentum* var. *Roma* cotyledons

The same plant expression vectors used to transform tobacco plants—pPCV701-AB, pPCV701-*luxFP1-B*, and pPCV701-*luxAB*—were used to transform tomato plants. As

before, the pPCV701-*luxAB* plasmid was a positive control for all transformation methods.

Agrobacterium-mediated transformation and regeneration of several tomato *L. esculentum* cultivars were first reported by McCormick et al., (1986). However, tomato is still considered to be more difficult to transform than other species such as *N. tabatum* and *Petunia hybrida*. The successful transformation widely depends on (1) the specific cultivars used, (2) the *Agrobacterium* strain, (3) the antibiotic selection, and (4) even the individual performing the procedures.

The first tomato transformation experiments were unsuccessful. They used MS medium containing 0.5 mg/L NAA and 0.1 mg/L BAP to initiate the regeneration of sliced cotyledons after they had been incubated with *Agrobacterium* donor strain. Green calli started to form after two days incubation in MS medium. Upon development of root initials from the cotyledons at 7-8 days, they were transferred to D1 medium supplemented with kanamycin. At 20 days of incubation, however, there was still no indication of shoot development.

To address this problem, a different protocol was evaluated. The sliced cotyledons were immediately transferred to D1 medium containing kanamycin after incubation with the *Agrobacterium* donor strain. Green calli began to develop five days after incubation. Shoot initiation on D1 + kanamycin plates occurred fifteen days after transfer. When the regenerating shoots were transferred to D2 medium (D1 medium with 0.1 mg/L zeatin) containing 100 mg/L kanamycin, a decrease was observed in the elongation rate of shoots. Root initiation was attempted using MS medium containing 50 mg/L kanamycin. Less than 10% of shoots developed root system under this condition, showing that kanamycin was inhibitory for the formation of a root system.

From these findings, a decision was made to use kanamycin containing D1 medium consistently during shoot development and to use MS medium without kanamycin selection

for root initiation and development (Methods 3.5). The whole process of transformation from first day incubation with *Agrobacterium* to obtaining fully developed plantlets (leaf system and root system) took about sixty days. The general steps and time requirements for the different developmental stages are summarized in Table 10. Fifteen plants were regenerated following transformation with pPCV701-*luxFP1-B* and seven plants were regenerated following transformation with pPCV701-AB. These plants were grown to maturity and maintained in the greenhouse.

2.4 Summary

The second objective of this project was to transform tobacco and tomato plants with the *B. subtilis* BCOADc genes. To achieve this objective, the A and B genes (encoding E1 α and E1 β subunits of BCOADc) were first subcloned into two different plant expression vectors and then transferred to plant tissues using *Agrobacterium*-mediated transformation. Both A and B genes were inserted into plant expression vector pPCV701 to produce vector pPCV701-AB. Just the B gene was inserted into luciferase expressing plant expression vector pPCV701-*luxFP1* to produce vector pPCV701-*luxFP1-B*. A third expression vector, pPCV701-*luxAB*, was used as a positive control. Following *Agrobacterium*-mediated transformation, the regeneration of tomato cotyledons proved to be more challenging than the regeneration of tobacco leaf disks (Table 11). Nevertheless, a sufficient number of both types of regenerated plants were obtained for further study.

B Gene

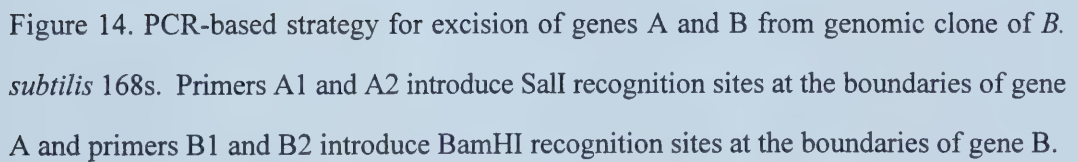


Figure 15. Analysis of DNA fragments produced by PCR using A1, A2, B1, and B2 PCR primers on agarose gels.

Lane A: A gene product (1009 bp).

Lane B: B gene product (1000 bp).

Lane M: λ /HindIII molecular weight markers.

Lane M₁₂₃: DNA ladder of 123 bp molecular weight markers.

M A B M₁₂₃



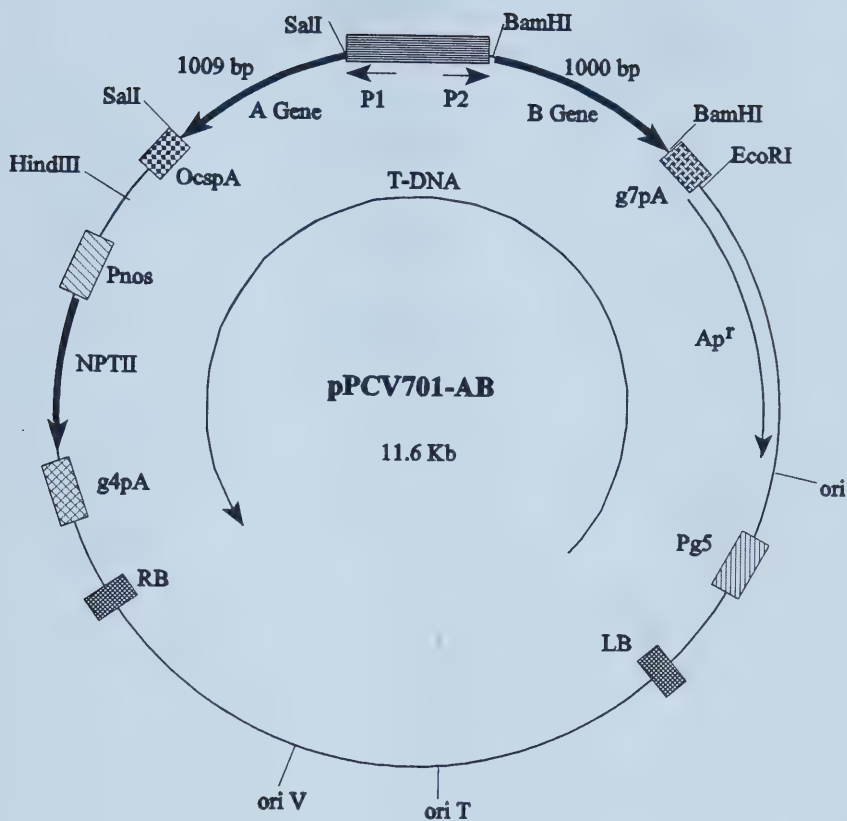


Figure 16. Restriction map of plasmid pPCV701-AB.

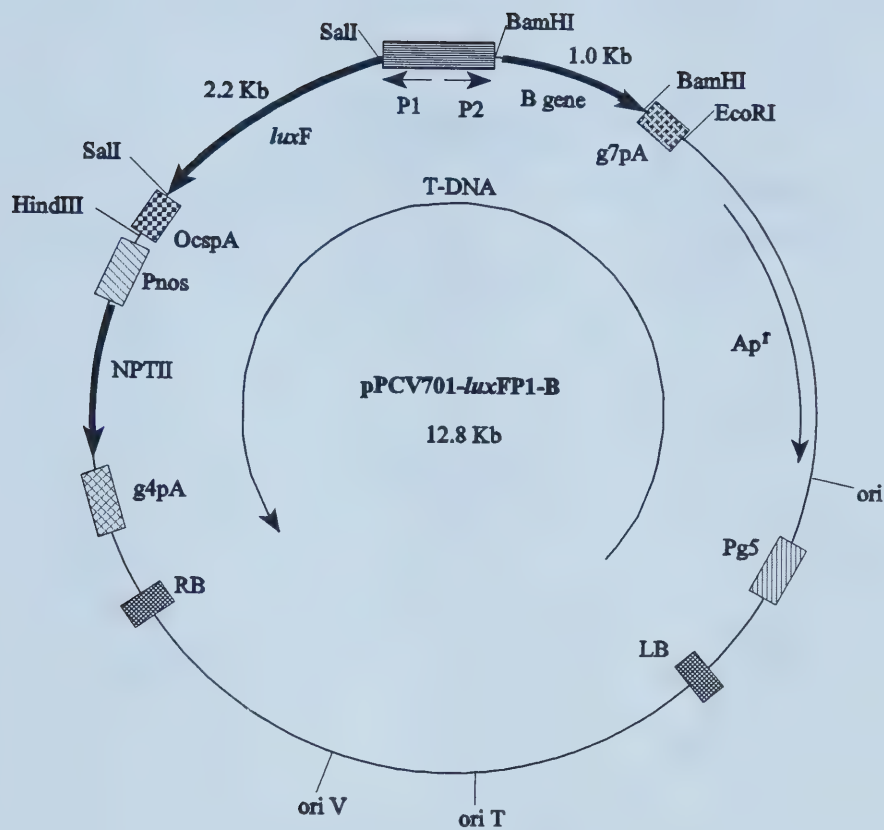


Figure 17. Restriction map of plasmid pPCV701-luxFP1-B.

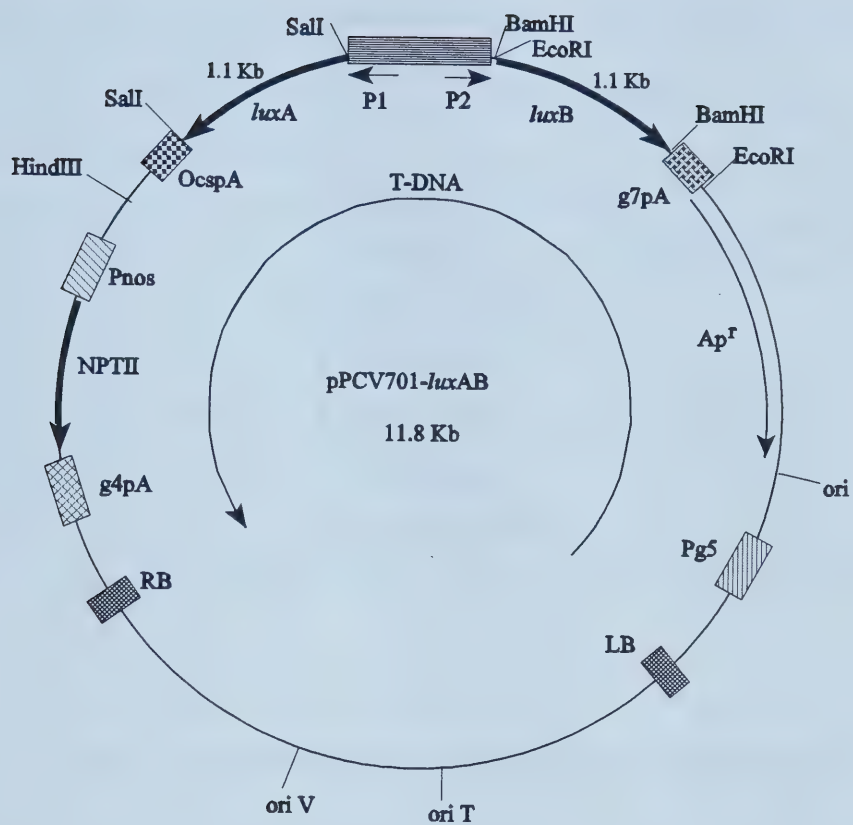


Figure 18. Restriction map of plasmid pPCV701-*luxAB*.

Table 10. Timeline for regeneration of transformed tomato cv. Roma cotyledons

TIME LAPSED (DAYS)	TIME AT EACH STAGE (DAYS)	DEVELOPMENTAL STAGE
1	-	Transformed cotyledons
3	2	Transfer to D1 + Km + Claf medium
8	5	Start to make green calli
13	5	Start shooting
50	30-40	Transfer to rooting medium (MS + Claf)
57	6-10	Start rooting
72	15	Move to greenhouse

Table 11. Comparison of tobacco and tomato regeneration studies

Plant Transformed	Plasmid Construct	Plants Regenerated
TOBACCO	pPCV701-AB	43
	pPCV701- <i>lux</i> FP1-B	25
	pPCV701- <i>lux</i> A B	22
TOMATO	pPCV701-AB	7
	pPCV701- <i>lux</i> FP1-B	15
	pPCV701- <i>lux</i> AB	3

3. Luciferase and Southern Hybridization Analyses of Transformed Plants

3.1 Luciferase assays of tobacco plant transformants

Bioluminescence was used to obtain preliminary evidence of plant transformation. Luciferase activity was first assessed *in vivo* on samples of leaves, stems, and flowers from regenerated tobacco plants transformed with pPCV701-*luxAB*. Figure 19 shows the low light image analysis of luciferase activity using plant tissues. Panel A shows the light emission from the induced leaf tissues; panel B displays light emission from the induced stem tissues, both measurements were made 24 hours after induction; and panel C exhibits the luciferase activity from fresh flowers of plant #5 under low light image equipment without hormone induction. (These video images were recorded photographically with the Polaroid "Screen Shooter" directly from the TV monitor.) The luciferase-mediated bioluminescence provided the first evidence that the plant expression vector containing luciferase A and B genes was successfully transformed and expressed in the transgenic tobacco plants. The bioluminescence of individual transgenic plants varied: tissues from plants #2 and #5 produced more bioluminescence than plants #1, #3, and #4 (in 20 minutes of photon counting). Transformed plant #6 did not exhibit any bioluminescence.

Quantitative analysis of luciferase reporter gene expression in these plants was performed by luminometry assay of extracts of base leaves, top leaves, and stems *in vitro* (Table 12). As in the *in vivo* evaluation above, Plant #2 and #5 (containing pPCV701-*luxAB*) exhibited the highest level of luciferase activity. Plant #6, again, exhibited the lowest level of luciferase activity. It was apparent that the stem tissues contributed the highest luciferase activity among all the samples suggesting a spatial pattern of foreign gene expression in tobacco. The stem tissue (with meristematic cells) may be the most efficient tissue for

induction of foreign gene expression using *mas* promoters in tobacco.

Luciferase activity was assessed next in tobacco plants transformed with pPCV701-*luxFP1-B*. Luminometry measurements were made for control and hormone-treated leaf tissues at different stages of plant development. A total of 22 plants (#2-#23) were investigated (Table 13). Leaf discs produced higher luciferase activity when plants were young compared to the same plants grown in the greenhouse for a longer period of time. Auxin-induced leaf disks exhibited higher luciferase activity. The same samples exhibited strong light emission after the addition of luciferase substrate decanal under the low light image analyzer before the luminometry assays (Figure 20). Plants which produced higher luciferase activity were maintained for further study. Plants with lower expression levels or no expression were discarded.

3.2 Luciferase assays of tomato plant transformants

Regenerated tomato plants transformed with plasmid pPCV701-*luxFP1-B* developed leaves and roots before moving them into the greenhouse. Seven days after acclimation in the greenhouse, all the plants had developed 3-4 leaflets. The bottom-most leaflets were harvested from each plant, the surface of leaves wounded, and the excised leaves placed on 2,4-D medium to activate the *mas* promoters. The luciferase activity of 1 cm diameter leaf discs of 13 regenerated tomato plants was determined by luminometry 24 hours after incubation (Table 14). Plants #1, #3, #5, #10 and #11 produced higher than 50 light units. Plants #8 and #9 were questionable. Other plants, #2, #4, #7, #12 and #13 did not show an increase in bioluminescence suggesting that they may not have received the plasmid containing the *luxF* gene.

Detection of pPCV701-*luxAB* gene product was more difficult because no significant luciferase activity was detected from induced leaf tissues at early stages. Information

previously obtained in our laboratories showed that callus, requiring high concentrations of auxin for growth, is the tissue that exhibits the highest *mas* promoter activity. Thus, calli from transformed plants can be useful in solving problems of low level expression. Calli from plants #1, #5 and #6 were raised from plant stems by culture on callus formation medium. Several calli were formed on the plates and light emission measured using luminometry (Table 15) and low-light imaging analysis (Figure 21).

3.3 Southern hybridization analysis of tobacco transformants

Plants transformed with pPCV701-*luxFP1-B* and exhibiting the highest levels of bioluminescence (Table 13) were subjected to Southern hybridization analysis using pBlue-B1 as the probe (Figure 22, A). Genomic DNA isolated from leaf tissues of eight plants (#4, #5, #9, #10, #11, #13, #14, and #20) were digested with the restriction enzyme EcoRI. A partial restriction map of pPCV701-*luxFP1-B* (Figure 23) indicates that a 2.81 Kb fragment containing gene B would be generated by digestion with restriction enzyme EcoRI. Calculation of the molecular weight of the bands which hybridize to probe (Figure 22, A) clearly shows that the signals obtained from the plant samples correspond to a 2.8 Kb EcoRI fragment. The untransformed control, SR1, produced no hybridization signal (as expected), failed to hybridize to the probe containing gene B. In addition, genomic DNA from plant #9, #11, #13 and #14 digested with BamHI and subjected to Southern hybridization analysis (Figure 22, B), confirmed the presence of the B gene encoding the E1 β subunit of BCOADc in tobacco plants.

Plants transformed with pPCV701-AB could only be evaluated by Southern hybridization analysis since they do not carry luciferase marker genes. Genomic DNA was prepared from regenerated tobacco plants #1, 2, 3, 4, 5, 6, 7 and 8 which had been transformed with the pPCV701-AB construct. The products of double EcoRI and HindIII

digestions of the genomic DNA were separated by agarose gel electrophoresis, transferred to nylon membranes (blots), and analyzed for hybridization to the probe, pBlue-B1 probe. All digests displayed a positive signal at 2.56 Kb, as expected, with the exception of plant #8. (Figure 22, C). The 2.56 Kb band is consistent with the restriction map of pPCV701-AB (Figure 24). Plant #8 is likely to be a spontaneous kanamycin resistant mutant rather than a transformed plant.

3.4 Southern hybridization analysis of transformed tomato plants

Southern hybridization analysis was performed on genomic DNA from plants transformed with pPCV701-*luxFP1-B* (Figure 25) and with pPCV701-AB (Figure 26). Genomic DNA samples from plants #1, #3, #5 and #10 transformed with pPCV701-*luxFP1-B* and exhibiting luciferase activity were digested with BamHI. Similarly, genomic DNA samples from plants #1, #2, #3, #4, #5, #6 and #9 transformed with pPCV701-AB were digested with BamHI. Blots were hybridized with purified B gene fragment labeled with ^{32}P -dATP. Figure 26 shows that plant #9 does not hybridize to the probe and, therefore, is probably a spontaneous kanamycin resistant mutant rather than a transgenic plant.

3.5 Determination of copy number for foreign genes in transgenic plants

Copy number of integrated foreign genes in transformed tomato plants was also examined by Southern hybridization analysis. Recent studies suggest that transgene copy number can positively or negatively affect transgene expression (Hobbs et al., 1993; Grevelding et al., 1993). T-DNA can integrated more than once into the same plant genome during the transformation event. Although single integration events are most often observed, multiple integration at the same site and at different sites has also been reported. Multiple T-DNA insertions have been found as direct repeats in head-to-tail arrays (e.g., RB/LB), as

inverted repeats in head-to-head arrays (RB/RB or LB/LB inverted repeat structures), or in even more complex structures. Individual hybridization band signals are expected upon a single T-DNA insertion. These signals vary in size depending on the fixed restriction sites in the T-DNA and the nearby random restriction sites in the plant genome. Multiple T-DNA insertions result in a hybridization pattern including two or more signals; certain inverted repeat type insertions (see below) result in a predictable hybridization pattern.

Southern blot hybridization performed to assess the copy number of A and B genes inserted into plant genomic DNA suggested that both single and multiple insertions occurred in the transgenic plants generated by this project. Digestion with the restriction enzyme HindIII was chosen because of the unique location of a HindIII cleavage site in the pPCV701 T-DNA vector. The probe was either purified A gene or B gene. DNA fragments which hybridize to the probe should produce hybridization signals larger than 5.96 Kb in digests from pPCV701-AB construct containing plants; hybridization signals should be greater than 7.53 Kb in the case of pPCV701-*luxFP1*-B construct containing plants (Figure 27, A & B). Plants #1 and #4 transformed with pPCV701-AB produced single hybridization signals; plants #3, #5, and #6 transformed with the same plasmid appeared to contain more than one T-DNA insert (Figure 28, A). Transgenic plant #2 transformed with pPCV701-AB appears to contain a single T-DNA insertion; however, the strong band (9.56 Kb) may be the result of one or more repeats in head-to-tail arrays (Figure 27, C). Plant #1 transformed with pPCV701-*luxFP1*-B produced a single hybridization signal; plants #3, #5, and #10 transformed with the same plasmid appeared to contain more than one T-DNA insert (Figure 28, B).

3.6 Summary

The third objective of this project, to confirm the presence of the BCOADc genes,

was accomplished by Southern hybridization analysis of genomic plant DNA using probes for BCOADc genes A or B. Moreover, differences in the strengths of hybridization signals and the molecular weights of DNA fragments exhibiting hybridization signals, the copy numbers of the A and B genes appeared to vary between independent transgenic plants. Lastly, the detection of luciferase activity *in vivo* and *in vitro* provided preliminary information regarding the differential expression of foreign genes in different plant tissues upon induction of the *mas* dual promoters.

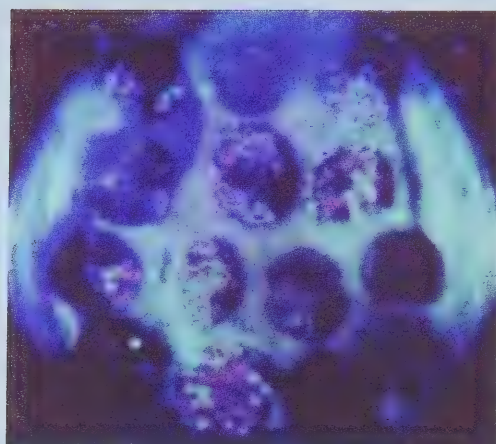
Figure 19. Low-light image analysis of luciferase activity from tobacco tissues containing *lux* A and B genes under the control of the *mas* dual promoter.

Panel A: Induced leaf tissues from 6 plants, exposed for 20 minutes. Top two rows are the leaf discs from plants #1 (left two), #2 (middle two), and #3 (right two). Bottom two rows are the leaf discs from plants #4 (left two), #5 (left middle two), and #6 (right middle two), and control (right two).

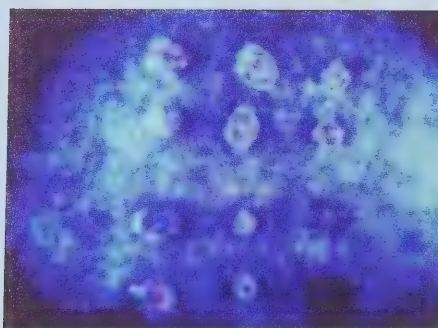
Panel B: Induced stem tissues from 6 plants, exposed for 20 minutes. Samples are organized the same as above.

Panel C: Three fresh flowers from plant #5, exposed for 30 minutes in the presence of decanal vapor.

A



B



C

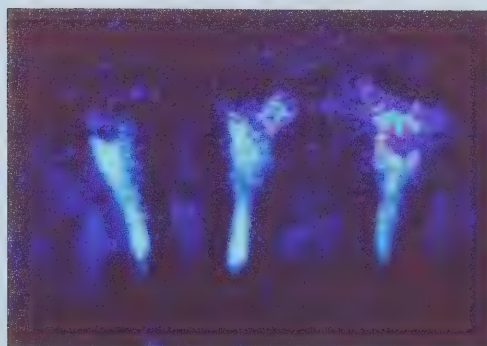


Table 12. Luciferase activity *in vitro* in regenerated tobacco plant tissues transformed with pPCV701-*luxAB*

Plant sample	Flower (LU)	Base leaf (LU)	Top leaf (LU)	Stem (LU)
1	0.2	81.4	148.3	1.9
2*	109.2	315.9	250.7	52.5
3	11.0	82.4	67.2	964.4
4	14.0	101.4	458.6	999.9
5*	38.8	1542.0	1286.0	6218.0
6	1.5	61.0	10.5	40.0
Control	0.0	2.2	0.0	1.7

* Samples show highest levels of *lux* gene expression.

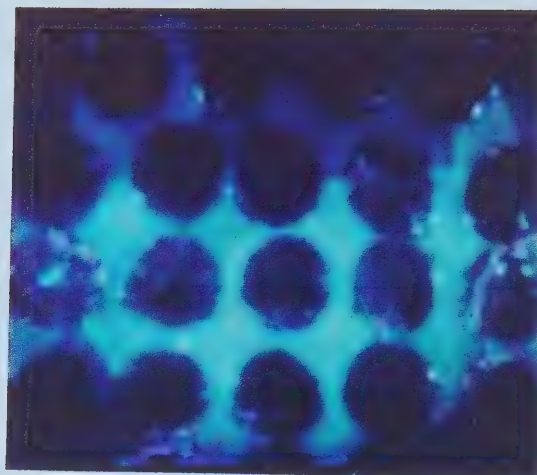
Table 13. Quantitative analysis of luciferase activity in regenerated tobacco plants transformed with pPCV701-*lux*FP1-B. Plant leaves were assayed 10 days after transfer into the greenhouse without induction.

Plant Sample	Light Unit/100mg tissue
Control	-0.5
2	7.0
3	1.1
4	11.7
5	9.6
6	2.0
7	3.1
8	10.4
9	3.2
10	6.1
11	10.8
12	2.7
13	2.9
14	5.0
15	2.7
16	1.3
17	6.7
18	1.8
19	6.1
20	1.3
21	5.4
22	4.0
23	0.1

Figure 20. (next page, A) Analysis of leaf discs from pPCV701-*luxFP1-B* transformed tobacco plants (as Table 13) by low light imaging before the assay in a Turner luminometer.

Figure 21. (next page, B) Low light image analysis of tomato calli regenerated from stems of pPCV701-*luxA* & B transformed tomato plants number 1 (left), 5 (right top), and 6 (right bottom). Exposure time: one minute.

A



B

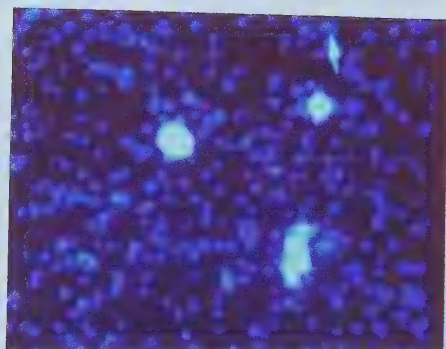


Table 14. Analysis of tomato leaves from regenerated plants transformed with pPCV701-*lux*FP1-B by luminometry. Sterile plants from light room were transferred into the greenhouse and assayed after seven days adaptation time. The *mas* promoters were induced by wounding of leaves and incubation of excised leaf tissues on 2,4-D medium one day prior assay.

Plant Sample	Light Unit (Full)/100 mg tissue
Control	0.2
1*	88.4
2	0.0
3*	79.3
4	0.2
5*	167.5
6	0.1
7	0.1
8	13.8
9	3.9
10*	86.7
11*	158.4
12	-0.4

Full: The Light Unit collected during the measurement. Control: non-transformed plant.
 * Plants selected as transformants for further analysis.

Table 15. Quantitative analysis of luciferase activity in calli of regenerated tomato plants (as Figure 21) transformed with pPCV701-*lux* A & B and assayed in luminometer. Calli were raised from stem segments taken from plants grown in greenhouse for 20 days after transfer.

Plant Sample	Light Unit (Full)
1	50.2
5	30.4
6	62.4

Figure 22. Southern hybridization analysis of tobacco transformants.

Panel A: Southern blot analysis of genomic DNA isolated from 701-*luxFP1-B* transformed tobacco plants # 4, 5, 9, 10, 11, 13, 14 and 20 using the linearized pBlue-B1 plasmid DNA as hybridization probe. The genomic DNA samples were digested with restriction endonuclease EcoRI. The arrow indicates the expected 2.81 Kb fragment released from plant genomic DNA by EcoRI and which hybridized with the probe. Lane M is the linearized pBlue-B1 plasmid DNA as a positive control.

Panel B: Southern hybridization of genomic DNA isolated from transformed tobacco plants number 9, 11, 13 and 14. The DNA samples were digested with restriction enzyme BamHI so that the entire B gene was released from the plant genomic DNA. The purified B gene fragment was used as the probe.

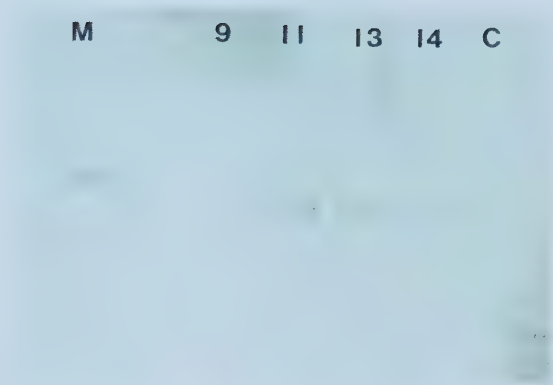
Panel C: Southern hybridization of genomic DNA isolated from tobacco plants transformed with pPCV701-AB. The linearized pBlue-B1 DNA was used as the probe. Genomic DNA samples were double-digested with restriction enzymes EcoRI and HindIII. Lane M indicates the pBlue-B1 DNA hybridization as a positive control.

A

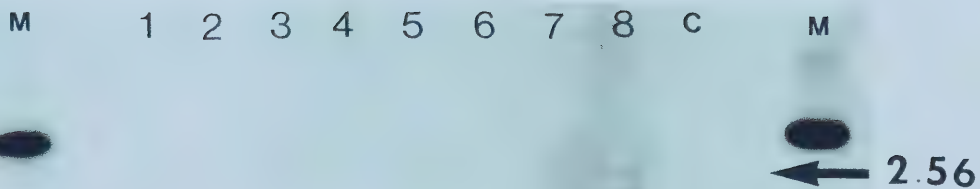


B

M 9 11 13 14 C



C



T-DNA of pPCV701-luxFP1-B

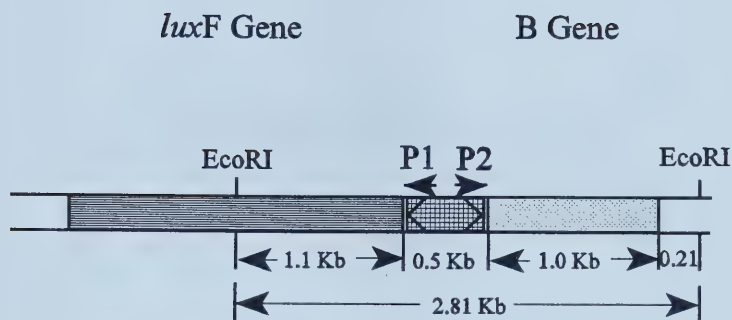


Figure 23. Restriction map of pPCV701-*luxFP1-B* plasmid DNA P1- P2 promoter region: restriction enzyme EcoRI releases a 2.81 Kb fragment.

T-DNA of pPCV701-AB

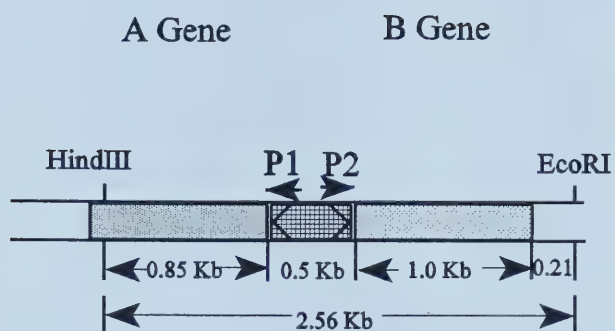


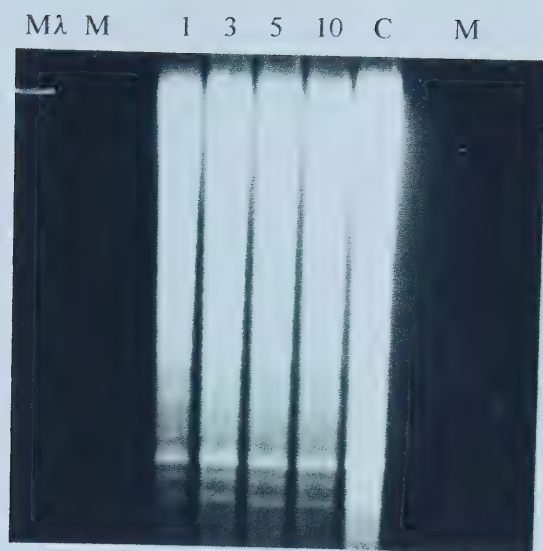
Figure 24. Restriction map of the integrated DNA from pPCV701-AB. A 2.56 Kb fragment is released after digestion with HindIII and EcoRI enzymes.

Figure 25. Separation of genomic DNA fragments isolated from tomato plants transformed with B gene of BCOADc and the luciferase fusion gene on agarose gels (A), and Southern blot analysis of the same digests using B gene fragment as ³²P labeled probe (B).

Panel A: Lane M λ , λ /HindIII molecular weight marker; lane M, purified B gene fragment 10 pg as a positive control; lane 1, 3, 5 and 10 are the genomic DNA samples isolated from transformed plants #1, #3, #5 and #10; lane C, DNA isolated from a nontransformed plant. Five DNA samples were digested with BamHI.

Panel B: Southern hybridization of above samples using purified B gene fragment as a probe.

A



B

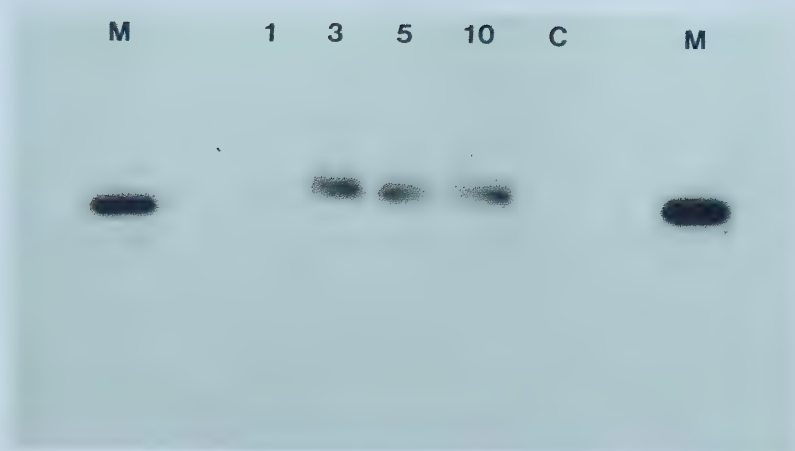


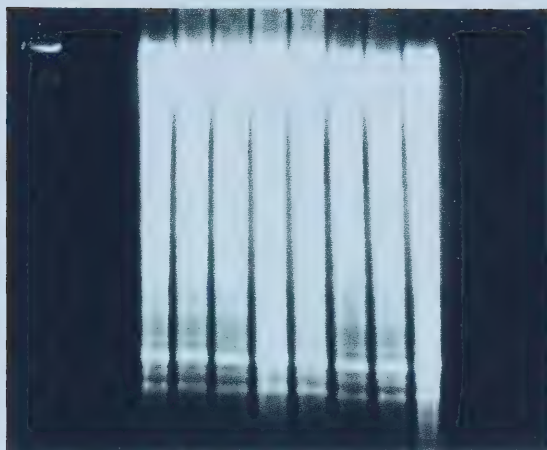
Figure 26. Separation of genomic DNA fragments isolated from tomato plants transformed with the A and B gene of BCOADc on agarose gels (A), and Southern blot analysis of the same digests using B gene fragment as ^{32}P labeled probe (B).

Panel A: Lane M λ , λ /HindIII molecular weight marker; lane M, purified B gene fragment 10 pg as a positive control; lane 1, 2, 3, 4, 5, 6 and 9 are the genomic DNA samples isolated from transformed plants #1, #2, #3, #4, #5, #6 and #9; lane C, DNA isolated from a nontransformed plant. Eight DNA samples were digested with BamHI restriction enzyme.

Panel B: Southern hybridization of above samples using purified B gene fragment as a probe.

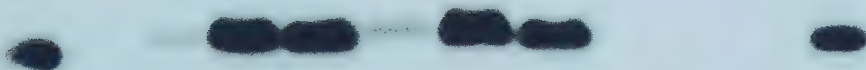
A

Mλ M 1 2 3 4 5 6 9 C M

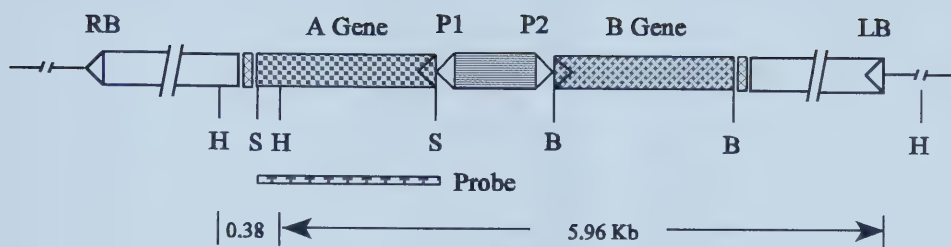


B

M 1 2 3 4 5 6 9 C M



A pPCV701-AB



B pPCV701-*luxFP1*-B

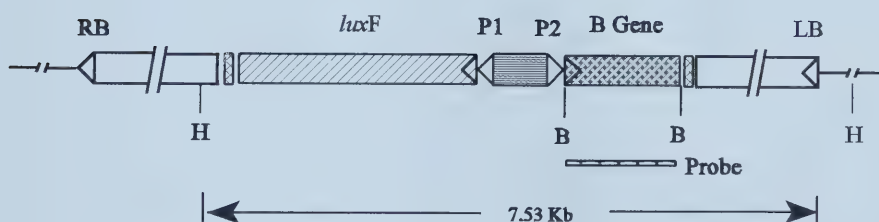


Figure 27. Restriction map of the A and/or B gene containing T-DNA inserts in tomato plant genomes. A, pPCV701-AB construct; B, pPCV701-*luxFP1*-B construct; C, head-to-tail organization of pPCV701-AB insert in transformed plants.

C Head-to-Tail Organization of Two pPCV701-AB Inserts

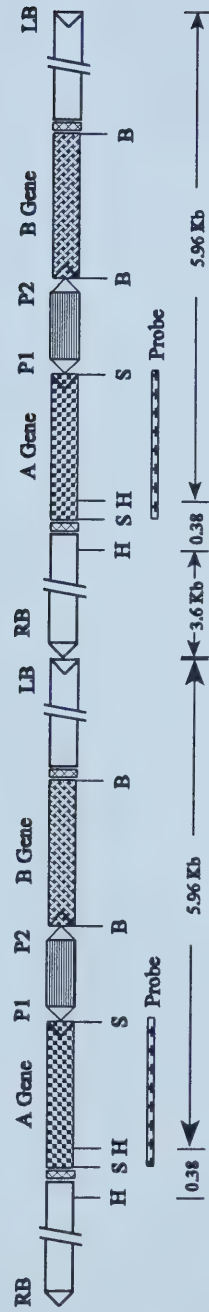
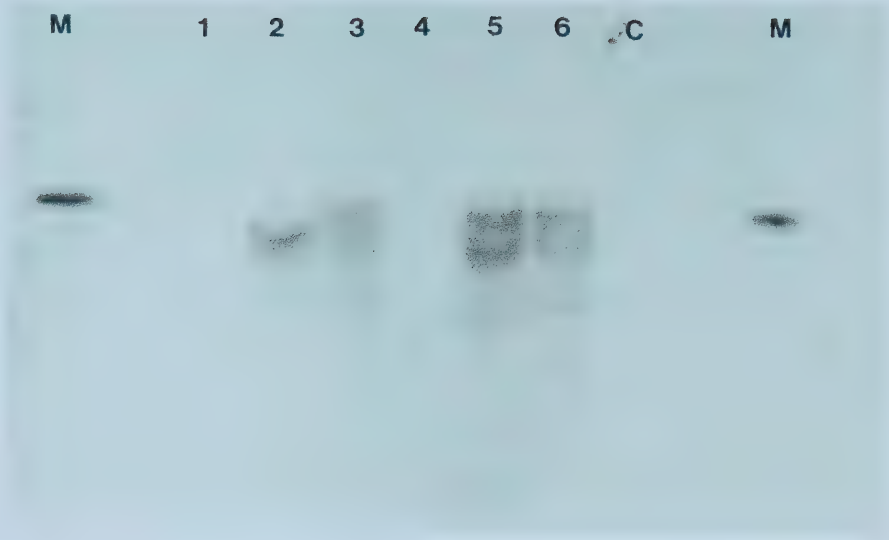


Figure 28. Determination of the copy number of transgenes in tomato transformants.

Panel A: Determination of the copy number of T-DNA inserts in six (1-6) independent transformants which contain insertions of the A and B gene. C indicates hybridization of probe to DNA from an untransformed plant (negative control); M is the plasmid pPCV701-AB position control.

Panel B: Determination of the copy number of T-DNA insert in four (#1, 3, 5 and 10) independent transformants transformed with B gene. C indicates DNA digest from an untransformed plant; M is hybridization of the probe to the purified B gene fragment as a positive control.

A



B



4. Transcriptional Analysis of Luciferase and BCOADc Transgenes in Transformed Tomato Plants

4.1 Preliminary experiments

Transcriptional analysis of A and B genes encoding E1 α and E1 β subunits of BCOA decarboxylase was carried out by using transformed tomato plant material. Total RNA was isolated from tomato fruit tissues of the pPCV701-*luxFP1*-B transformed tomato plants #1 and #10 and of the pPCV701-AB transformed tomato plants #1, #3 and #5 using hot-phenol extraction (Figure 29). RNA samples were examined by Northern hybridization using ³²P end-labelled primer #14 (for the presence of the A gene transcript) and #21 (for the presence of the B gene transcripts). When no positive signals were obtained by this procedure, alternative methods were employed to detect these mRNAs. Primer extension was successfully used to detect transcripts from BCOADc genes A and B, and RNase protection assays were used to detect transcripts from the *luxF* fusion gene.

4.2 BCOADc A and B gene messenger RNA transcription in tomato

Primer extension reactions were carried out using primers #14 and #21 and total RNA from transformed plants as the templates. The resulting cDNA products from reverse transcription are diagrammed in Figure 30. When primer #14 is used, a reverse transcript of 293 nucleotides is expected from gene A; when primer # 21 is employed, a reverse transcript of 205 nucleotides is expected from gene B. Primer extension experiments were performed for transgenic tomato plants #1, #3, and #5 transformed with BCOADc A and B genes (Figure 31). From 15 μ g of total RNA, plant #1 produced the strongest cDNA signals from both the A and B gene dependent primer extension reactions compared to plants #3 and #5. The relative amount of messenger RNA transcription from A and B genes is inferred from

the relative strength of the cDNA signals. Thus, plant #1 exhibits the highest level of A and B gene transcription. Primer extension experiments were also performed for transgenic tomato plants #1 and #10 transformed with the BCOADc B gene and the *luxF* gene (Figure 32). Transgenic plant #10 (containing multiple copies of the B gene) produced a stronger cDNA signal than transgenic plant #1, which carried single copy of the B gene. These findings suggest that multiple transgene inserts in *luxF*-B transformants produce more messenger RNA transcripts than single transgene inserts in the tomato genome.

Winter et al. (1984) have determined approximate start points (+1) of transcription of the dual *mas* promoter, P1 and P2 promoter (Figure 33). Heavy dotted lines mark the uncertainty of the nucleotides involved in the start of transcription; arrows show the precise starting point of transcription. Data obtained from primer extension experiments of *luxF*-B containing plants confirmed that the start point (+1) for the P2 promoter is at the 76th nucleotide downstream of BamHI restriction site.

4.3 *LuxF* messenger RNA transcription in tomato

Messenger RNA (mRNA) transcripts from the luciferase fusion gene (*luxF*) were analyzed by ribonuclease protection assays. This technique allows the detection but not the quantitative comparison of mRNA levels between different transgenic plants. A PCR fragment containing P1 promoter sequence (78 base pairs) and *luxA* 5' sequence (230 base pairs) was cloned downstream of the T7 promoter in plasmid pBluescript KS+/- . This construct named pP1-*luxBS* (B. Ayre, unpublished data) was used to produce an antisense RNA strand by T3 polymerase as a riboprobe. Total RNA containing *luxF* mRNA was prepared from pPCV701- *luxF*-B transformed plant #10. Figure 34 shows the gel electrophoresis of RNase protected fragments from *luxF*-B transgenic plant #10 as compared to an untransformed plant control. The hybridizing fragment has the same size as the

antisense *luxF* probe (310 bp), indicating the presence of the *luxF* mRNA by the plant cells. The hybridizing fragment from the positive control is smaller, since it hybridizes to the transcript of a *luxF* gene driven by CaMV 35S promoter, resulted in a protection fragment of 230 bp in length.

4.4 Summary

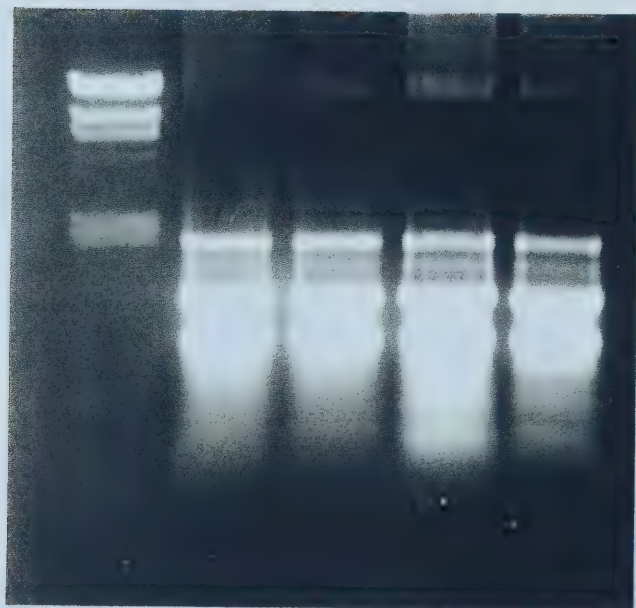
The fourth objective of this project: to carry out messenger RNA analysis of the BCOADc gene and *lux* gene transcription in transgenic tomato plants, was achieved using primer extension technique and RNase protection assay procedures. The data showed that the A, B genes and *luxF* gene were successfully transcribed in the transformed tomato tissues. Transcriptional levels varied among independent transformants.

Figure 29. Separation of total RNA isolated from fresh green fruits of transformed tomato plants in RNase free agarose gels. The samples were loaded onto agarose gels after pretreatment with DEPC and stained with ethidium bromide.

Lanes A-D: 5 µg of total RNA

M: λ/HindIII molecular weight markers.

M A B C D



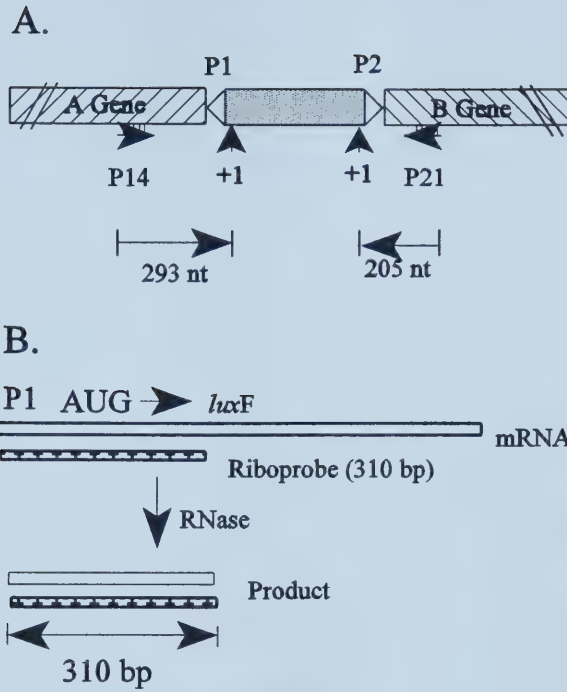


Figure 30. Transcriptional analysis of transgenes by primer extension and RNase protection assay. A: Detection of transcripts of the A and B genes in transformed tomato plants by primer extension. P14 is primer #14 complementary to mRNA of the A gene and results in a reverse-transcribed cDNA fragment of 293 bp; P21 is primer #21 complementary to mRNA of B gene and results in a reverse transcribed cDNA fragment of 205 bp. B: Detection of mRNA of luciferase fusion gene from *luxF*-B transformed tomato plant. The riboprobe is indicated by a bar underneath the *luxF* transcript (from Ayre, unpublished data).

Figure 31. Primer extension using total RNA isolated from the AB gene containing transformed tomato plants #1, # 3, and # 5.

C: RNA samples isolated from an non-transformed tomato plant.

P14: Reactions carried out using P14 primers for the detection of A gene transcripts.

P21: Reactions carried out using P21 primers for the detection of B gene transcripts.

[Arrows point to the cDNA products of the A gene (293 bp) and the B gene (205 bp)].

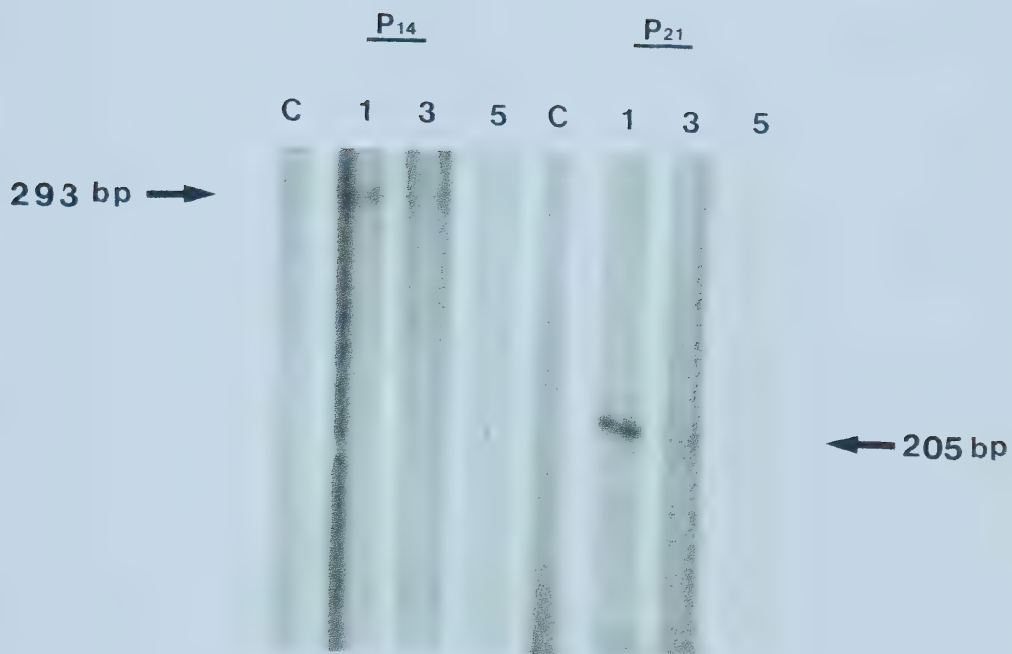


Figure 32. Primer extension using total RNA isolated from *luxF*-B gene containing transformed tomato plants #1 and #10 and P21 primer.

C: Total RNA isolated from an untransformed tomato plant.

(Arrows indicate the location of the cDNA products).

M: Molecular weight marker (base pairs).

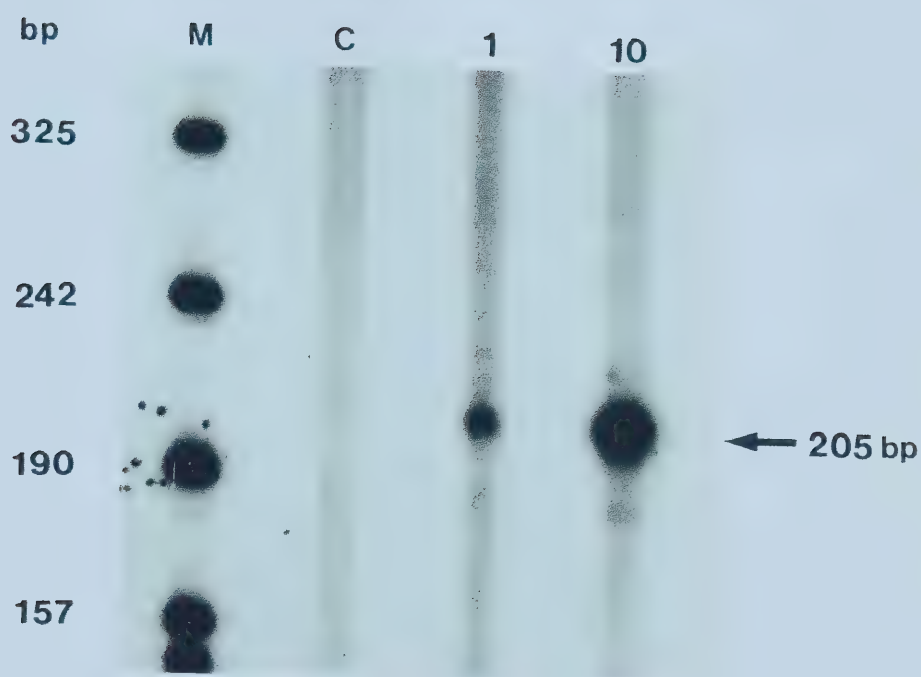


Figure 34. Ribonuclease protection assay.

M: Molecular markers of pBR322 digested with *Hinf*I. Bands from top to bottom are at the length of 1631, 517/506, 396, 344, 298, 221/220, 154 base pairs.

C: RNA isolated from an untransformed plant.

10: RNA isolated from *luxF*-B gene containing transformed tomato plant #10.

P: RNA isolated from a Poplar plant containing the *luxF* gene driven by CaMV 35S promoter.



5. Differential Expression of Luciferase in Leaf, Flower and Fruit During the Development of Transformed Tomato

5.1 Plant growth following transformation

Following transformation with plasmid pPCV701-*luxFP1-B* and regeneration, tomato plantlets (*L. esculentum* var. Roma) were grown in the light room until roots and shoots developed. Then, they were moved into the greenhouse and were grown in 8-inch pots (Methods 17.4). The plants were indeterminant and reached heights of 60-100 cm. The development of a mature fruit from a flowering stage took 4-6 weeks in winter and 3-4 weeks during the summer. A typical fruit was normally egg-shaped, 6 x 4 cm in size, and weighed about 50 grams. Figure 35 shows the growth morphology of the tomato plants at eight months after transformation. No major differences in morphology were observed between untransformed and transformed plants grown under greenhouse conditions.

5.2 Luciferase gene expression in vivo

Luciferase-mediated light expression (photon emission) was monitored in leaves and flower parts from three independent transformed plants #3, #5, and #10. First, measurements were made of terminal and lateral leaflets (Figure 36, A). Photon emission was monitored for 15 minutes in the presence of the substrate decanal. High levels of bioluminescence were seen in the tip of the leaves which gradually decreased toward the base of the leaflets. Second, the photon emission of flowers from the three transformed plants was compared with flowers of an untransformed tomato plant (Figure 36, B). The flowers from three transformed plants and one untransformed plant were dissected into sepals, petals, stigma and style, and stamens, and monitored using the low-light image analysis system. Strong light emission was observed five minutes after exposure to decanal (Figure 36, B). These

results demonstrate that the *mas* promoter is strongly activated in transformed plants, whereas the nontransformed plant, since it did not receive the luciferase gene, did not show light emission.

Tomato fruits from these transformed plants were also analyzed throughout development and ripening. Tomato fruits were cross-sectioned into 6-7 slices and four slices were placed side by side in 9-cm diameter petri dishes on water saturated filter papers. The slices were incubated at room temperature for specified time periods.

Green fruits from transformed plant #1 were monitored for changes in promoter activation 36 hours and 50 hours after cutting the slices. The result of a one minute light collection is displayed in Figure 37 A. The photon image was superimposed upon the video image of the tomato slices to allow correlation with light emission regions of the tissues. The luciferase activity is shown in picture (Aa) concentrated in the placenta area, the activity increased up to 50 hours after cutting (Ab). Green fruits from transformed plant #3 were monitored for one minute with the photon collecting camera at 20 (Ba), 36 (Bb), and 50 (Bc) hours after cutting (Figure 37 B). The same pattern of luciferase expression was obtained as described for plant #1. Fifty hours after slicing, the luciferase activity already began to spread into the fruit tissues through carpels to endocarp regions. Figure 37 C shows the luciferase activity measured from green fruit of #5 plant at 0 time (Ca) and 20 (Cb) hours after cutting. Luciferase expression increased dramatically after 20 hours. The results from all three independent transformed plants suggest that luciferase expression in green fruits is the highest at the placenta regions. In addition, the expression levels of luciferase (under the control of the *mas* promoter) increase dramatically with time (up to 50 hours) after the tissues are wounded by cutting.

In contrast to green fruits, fully mature red fruits exhibited higher levels of luciferase gene expression and an equal distribution of luciferase activity in the placenta, carpel, and

pericarp regions (data not shown). In addition, it was found that luciferase activity was always higher in leaflets, flowers, green and red fruits of plants transformed with pPCV701-*luxFP1-B* (#1, #5, #10) than in plants transformed with pPCV701-*luxAB* (#1, #5 and #6). This difference was independent of the method used to induce the *mas* promoter (wounding versus incubation with auxin). The reason for the difference has not yet been determined.

The induction of the *mas* promoter by plant growth hormones such as auxin (Langridge et al., 1987) suggests that changing auxin levels during development may result in temporal and spatial differences in luciferase activity in transformed tomato plant tissues. Several laboratories have suggested that auxin production increases in early seed development and decreases during seed maturation. In immature green fruit tissues, the plant embryos produce free auxin in the placental region. As the fruit matures and grows in size, levels of auxin increase in the pericarp tissues. Finally, at the ripe (red) stage, auxin reaches very high levels and spreads throughout the entire fruit (Langridge et al., 1987). Consistent with these developmental changes in auxin production, *mas* promoter driven luciferase gene expression in green fruits from var. Roma was greater in the older fruit than in the younger fruit (Figure 38). Furthermore, several seeds had been isolated from the fruit tissues and opened by cutting. Luciferase activity was evident after 30 minutes of photon collection. The bioluminescence from seeds was lower than expected and lower than that observed from fruit tissues (data not shown). Further experiments are necessary to compare bioluminescence in seeds and placenta tissues during different developmental stages, and to correlate this gene expression with relative auxin levels in these different tissues. It is not known whether wounding induces the production of auxin or wounding induces *mas* promoter activation through another mechanism.

5.3 Influence of temperature on mas promoter induction by mechanical wounding of

plant tissues

The quantitative analysis of luciferase activity expressed from the *mas* promoter in the green fruits of tomato plants transformed with pPCV701-*luxFP1-B* demonstrates that induction by wounding is temperature dependent. Green fruits about 3 cm in diameter were harvested and sliced into 5 mm thick sections. The sections were then cut in half, one half was incubated at 22°C (room temperature) and the other at 4°C (Figure 39). Luciferase enzyme activity was monitored *in vitro* every 24 hours for four days using a luminometer. At each timing point, a tissue sample (~ 100 mg) was excised from the same position on each slice. All luminometer readings were based on the Light Units from 100 mg fruit tissue. An assumption was made that the total protein in 100 mg of fruit tissues from all fruits of the same age was similar.

Figure 40 presents the luciferase activities obtained from wounded fruit tissues after 24 hour incubation at 22°C compared with incubation at 4°C. All fruits from three transformed plants (#1, #5 and #10) showed higher luciferase enzyme activity after incubation at 22°C as compared to 4°C. Plants #5 and #10 exhibited approximately 20 times more luciferase activity at the higher temperature. Luciferase activity had increased in all tissue samples at both temperatures 48 hours after wounding (Figure 40). A summary of luciferase activity over the entire four day period for transformed plant tissues incubated at both temperatures (Figure 41) illustrated the influence of temperature on induction of the *mas* promoter by wounding. Tissue slices incubated at 22°C had their highest activity on day two; in contrast, tissue slices incubated at 4°C exhibited continuous increases in luciferase activity (in tissues two out of three independent transformed plants).

To further study the effect of temperature on *mas* promoter activation after wounding, a temperature shift experiment was carried out (Figure 42). Luciferase activity in plant tissues incubated at either 22°C or 4°C for 24 hours after cutting was measured quantitatively

using a luminometer, then the tissue slices were shifted to the opposite temperature and incubated for an additional 24 hours. Tissue slices switched from low temperature to high temperature displayed dramatically increased enzyme activity from all three plants tested. The tissues switched from high temperature to low temperature also showed an increase in luciferase activity in plant # 5 and #10. The activity from plant #1 however, decreased after switching the temperature from 22°C to 4°C. These results suggest that wounding at 22°C can induce the *mas* promoter faster than wounding at 4°C. Once the promoter is activated in the tissue, however, it cannot be inactivated at the lower temperature (4°C) .

5.4 Summary

The results presented in this section suggest a spatial and temporal expression of luciferase i.e., luciferase activity varied in leaf, flower, and fruit tissues throughout development. Therefore, *mas* promoter activation can be located through the detection of luciferase activity. Wounding can induce *mas* promoter activity to higher levels in plant tissues. This activation depends on the induction temperature after wounding.

Figure 35. Growth morphology of *L. esculentum* var. Roma. Plants were grown in the greenhouse (16 hours photoperiod, 22/20°C) for 4 months.



Figure 36. Expression of bacterial luciferase fusion gene in tomato leaves and flowers.

Panel A. Analysis of luciferase marker enzyme in leaves from independent transformants of greenhouse grown tomato plants. Fresh leaves were measured for 15 minutes under the low-light image camera in the presence of decanal.

a and b: The terminal leaflet (a) and lateral leaflet (b) from transformed plant #3;
c and d: The terminal leaflet (c) and lateral leaflet (d) from transformed plant #5;
e and f: The terminal leaflet (e) and lateral leaflet (f) from transformed plant #10.

Panel B. Expression of luciferase fusion gene in flowers of transformed tomato plants.

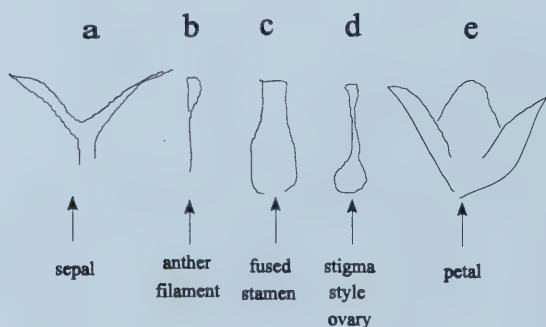
Row A: Flower from plasmid pPCV701-*luxFP1-B* transformed plant #3.

Row B: Flower from similarly transformed plant #5.

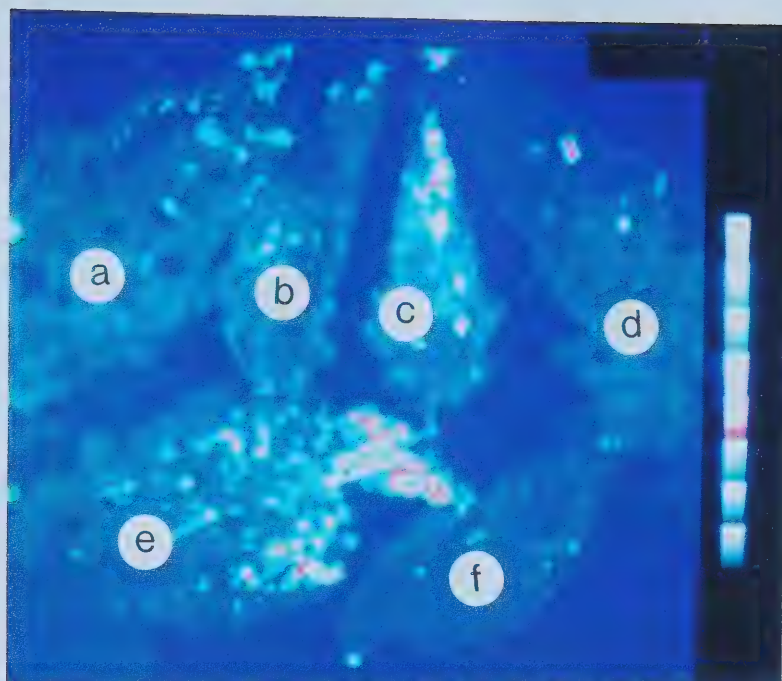
Row C: Flower from similarly transformed plant #10.

Row D: Flower from untransformed plant.

The flowers had been separated and arranged into five parts as showed below:



A



a b c d e

B

A

B

C

D

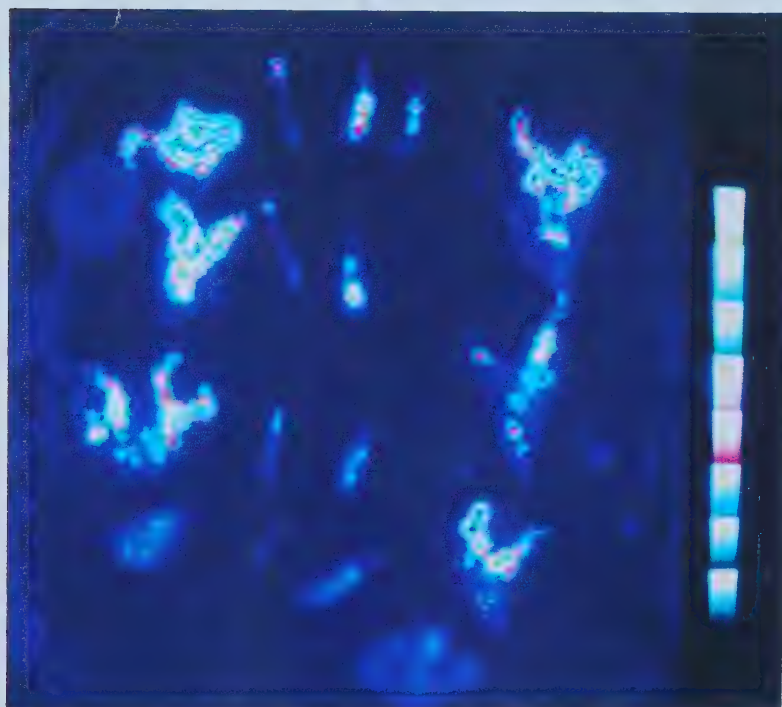


Figure 37. Analysis of *mas* promoter-luciferase fusion gene activation in green fruits of independently transformed tomato plants. Light emission pattern was measured in transformed plant #1 (A), #3 (B), and # 5 (C) at 0, 20, 36, and 50 hours after cutting.

Panel A: Detection of bioluminescence in fruits of transformed plant #1 by low light image analysis.

- a. Cross sections of a fruit measured 36 hours after cutting;
- b. The same fruit 50 hours after cutting.

Photon collection was carried out for 1 minute.

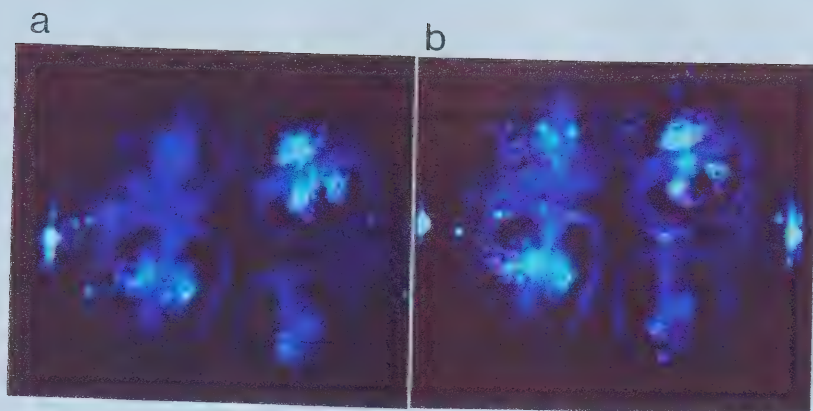
Panel B: Detection of bioluminescence in the fruits of transformed plant # 3 by low light image analysis.

- a. Green fruit detected 20 hours after cutting;
- b. 36 hours after;
- c. 50 hours after.

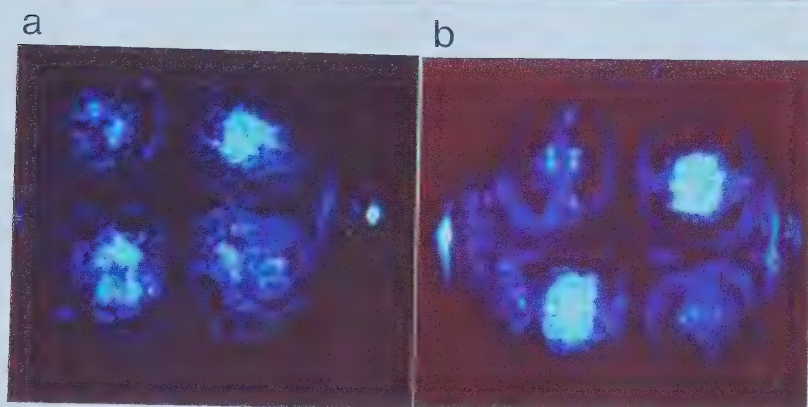
Panel C: Detection of bioluminescence in the fruits of transformed plant # 5 by low light image analysis.

- a. Green fruit measured immediately after cutting with 5 minutes photon collection time;
- b. 20 hours after with 1 minute photon collection.

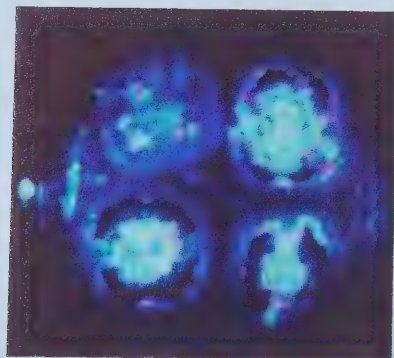
A



B



c



a

b

C

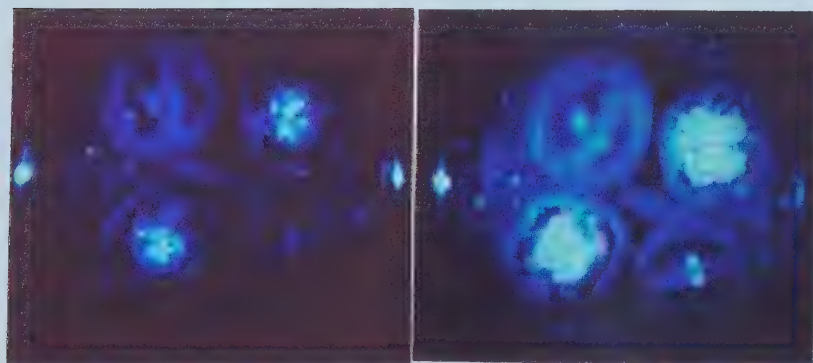


Figure 38. Localization of *mas* promoter activation by spatial and temporal expression of luciferase genes in dissected green fruits at different stages of development.

Panel A: Fruit samples used for the measurement. The top group (a) is a sectioned fruit from an untransformed plant. This fruit was cut into two halves vertically, one half was placed on the left, the other half was cross sectioned horizontally into five pieces and placed to the right of the first half. The other three groups (b, c, and d) are the tomato fruits from pPCV701-*luxFP1-B* transformed tomato plants. All samples were sectioned in a similar manner as above.

Panel B: The sample prepared above was placed in the photon collecting chamber, light was collected by low light imaging system for two minutes.

A

a

b

c

d



B

a

b

c

d

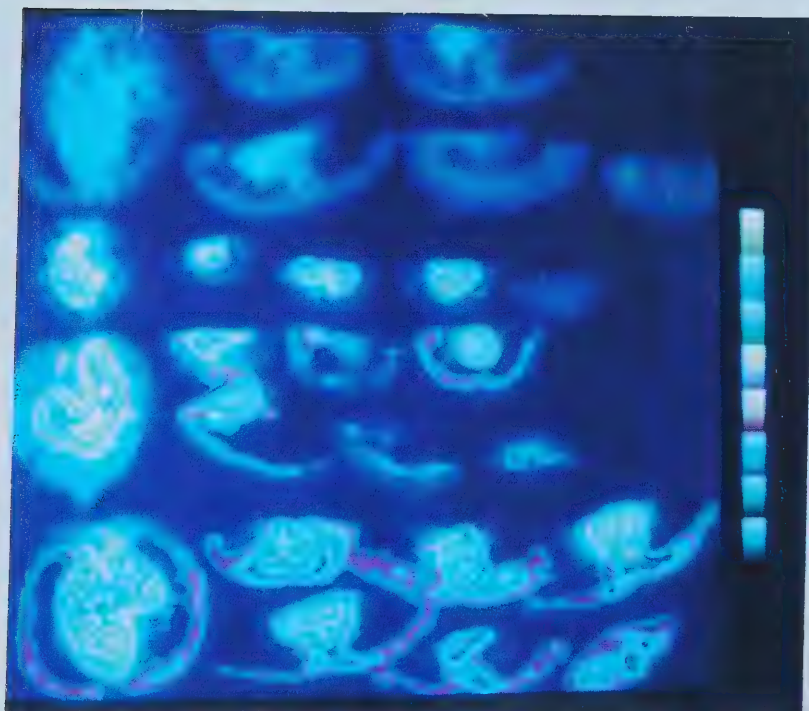


Figure 39. Sample preparation for the measurement of *mas* promoter activity. One fruit was cross sectioned into slices. Each slice was cut half, one half was incubated at 22°C, another half was incubated at 4°C.



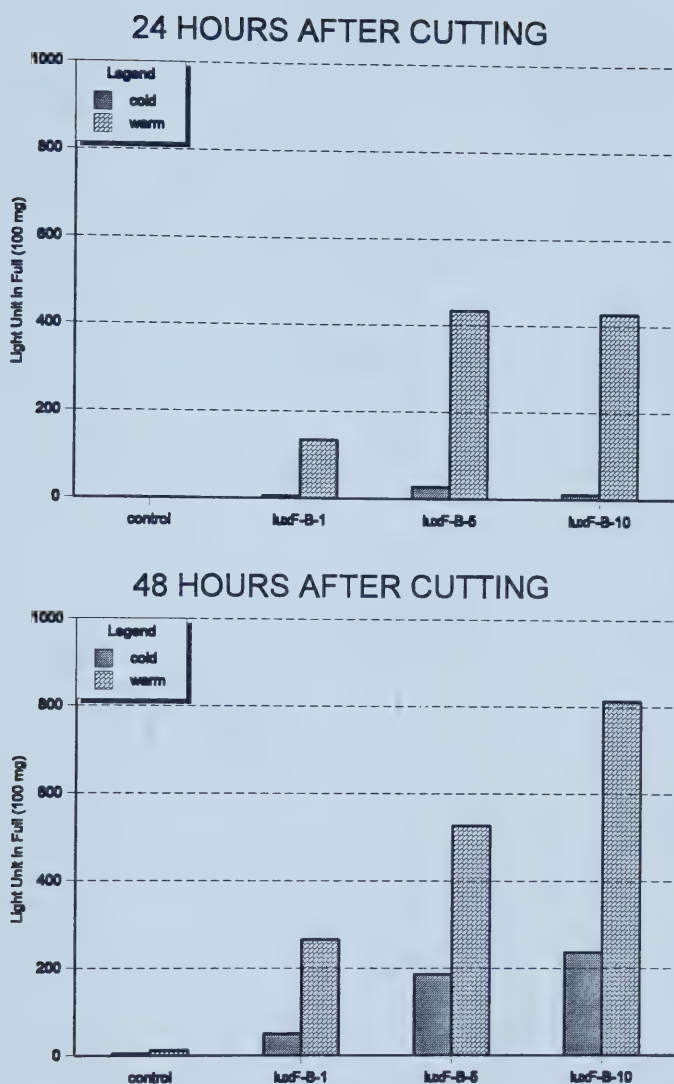


Figure 40. Luciferase activities (Light Unit/100 mg tissue) of tomato plants #1, #5 and #10 transformed with plasmid pPCV701-*luxFP1-B*. Measurements were made 24 and 48 hours after wounding the fruits and incubated at 4°C (cold) and 22°C (warm).

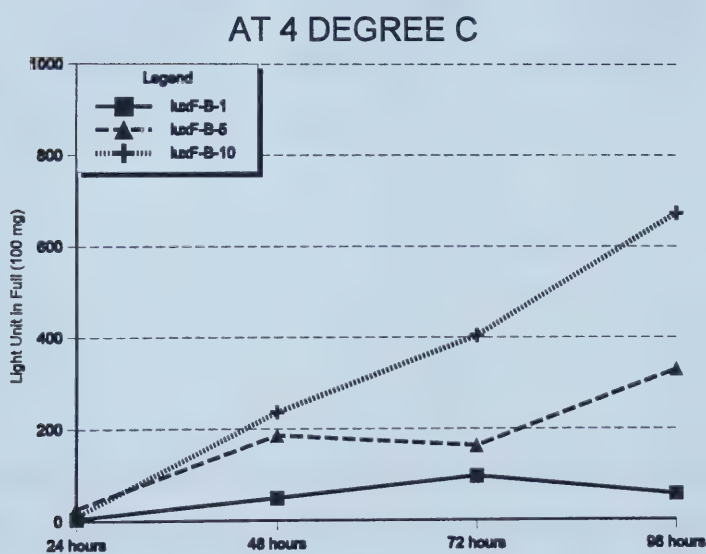
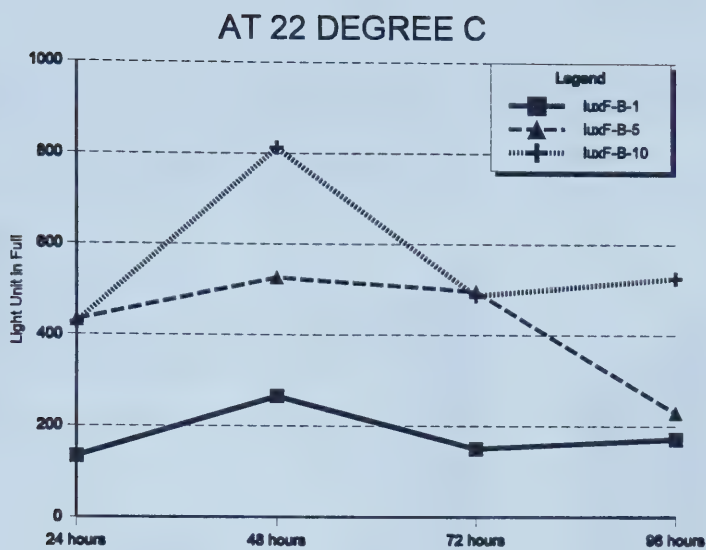


Figure 41. Summary of luciferase activities of tomato fruits from three *lucF-B* transformed plants #1, #5 and #10 after incubation at 22°C and 4°C for 1-4 days.

EFFECT OF TEMPERATURE SHIFT

ON MAS PROMOTER INDUCTION BY WOUNDING

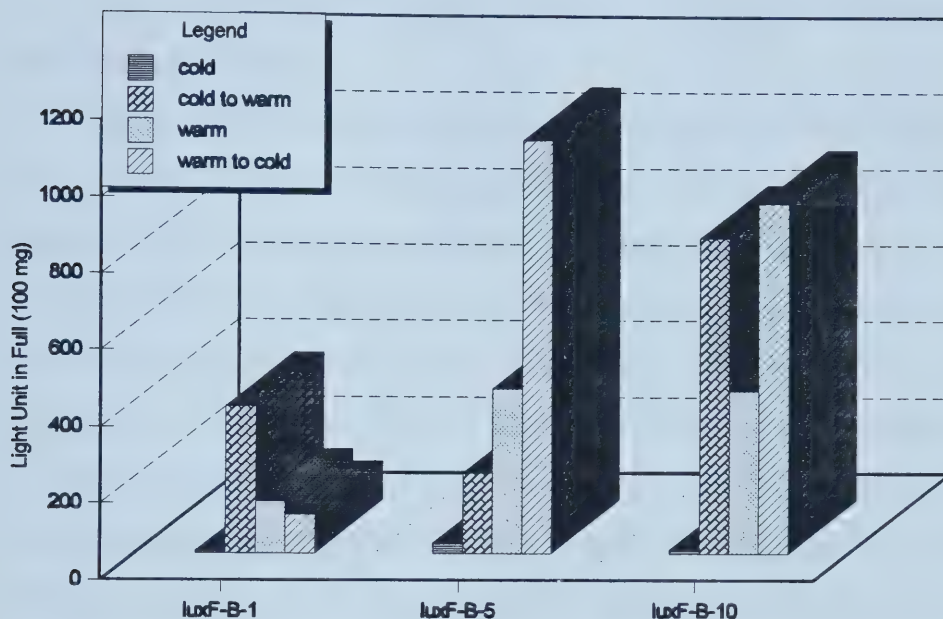


Figure 42. Luciferase activities of tomato fruits from *luxF-B* transformed plants #1 (*luxF-B-1*), #5 (*luxF-B-5*) and #10 (*luxF-B-10*) before and after a temperature shift. The tissue slices were incubated at 22°C (warm) or 4°C (cold) for 24 hours before being moved to 4°C (warm to cold) or 22°C (cold to warm) for an additional 24 hours.

6. Western (Immuno) Blot Analysis of Transgene Translation in Plant Tissues

6.1 *E. coli* production of recombinant BCOADc E1 α and E1 β subunit proteins for preparation of antibodies

The first step was to construct bacterial expression vectors for high yield production of the BCOADc E1 protein subunits encoded by the A and B genes. The initiation of translation events of a gene in bacteria requires a bacterial ribosome binding sequence which is located 5-10 nucleotides upstream of the start codon (AUG). This sequence is also called Shine-Dalgarno sequence or SD sequence. The SD sequence has been found conserved in *E. coli* strains as 5'-GGAGG-3' sequence. Moran et al., (1982) have reported that the same SD sequence has been observed in *B. subtilis* and other gram-positive organisms. Ribosome-binding sequences were identified in the A gene and B gene of E1 α and E1 β subunits of BCOADc (Figure 11). In order to synthesize E1 α and E1 β proteins in bacterial cells, SD sequences must be placed upstream of the translational start codon. This was achieved using PCR strategy described before (Methods 1.11); the only difference is that the 5' primer was designed so that the SD sequences were included in the primer sequences (see below).

Production of large amounts of foreign proteins in bacteria can be accomplished using several cloning systems, e.g. (1) the *E. coli* DLT101 expression system, (2) the pKK223-3 cloning and expression system, or (3) the Enterokinase Xpress™ system (Invitrogen Coop.). Each system has its own advantages. The *E. coli* DLT101 expression system produces a large amount of T7 RNA polymerase when the growing cells are induced by IPTG, so that genes under the control of T7 promoter can be expressed at a high level in this strain. Plasmid vector pBluescript II KS +/- and pT3/T7-19 are all suitable candidates. The plasmid pKK223-3 cloning and expression system has the advantage of possessing a

ribosome-binding site upstream of the multiple cloning sites which only requires that the start codon (ATG) of the foreign gene is located 5-10 base pairs downstream from the ribosome-binding site. This plasmid contains the strong hybrid *tac* promoter (*trp* promoter combined with *lac* operator) which is also induced by the addition of IPTG in the appropriate host. The Enterokinase Xpress™ system provides a convenient way to purify the target protein from the cell extracts by passing the extracts through a Ni charged resin column to purify the fusion protein, followed by Enterokinase cleavage to release the recombinant protein product.

The convenience of the *E. coli* DLT101 expression system contributed to its adoption for expression of A and B gene products. Three gene fragments were generated by PCR (Figure 43). A_{SD}B fragment covers A and B genes, with ribosome-binding sequences upstream using A_{SD} and B2 PCR primers; A_{SD} fragment includes A gene with ribosome-binding sequence upstream using A_{SD} and A2 PCR primers; B_{SD} fragment contains B gene with ribosome-binding sequence upstream using B_{SD} and B2 PCR primers. The restriction cloning sites were constructed so that SalI/BamHI was suitable for the A_{SD}B DNA fragment, SalI for A_{SD} and BamHI for B_{SD} DNA fragment. These three DNA fragments were amplified by PCR and subcloned further into pBluescript II KS +/-.

The transformed subclones containing the A_{SD}B fragment (2006 bp), the A_{SD} fragment (1023 bp) and the B_{SD} fragment (1015 bp) were analyzed by restriction mapping (data not shown). The correct DNA sequence of these subclones was confirmed using the ABI automated DNA sequencer. The data from two directions of DNA sequences from A_{SD}B, A_{SD}, and B_{SD} fragments matches the sequences obtained earlier from genomic DNA of *B. subtilis* 168s (data not shown).

Transformed *E. coli* DLT101 strains containing A_{SD}B, A_{SD} or B_{SD} plasmid constructs were generated by standard *E. coli* transformation procedures. The induction of foreign

protein synthesis was carried out in LB cultures by adding the inducer IPTG to a final concentration of 0.4 mM when the optical density of the cell culture reached OD₆₀₀ of 1.0 to 1.5. Total protein was then isolated from the cells and separated in SDS-polyacrylamide gels by gel electrophoresis (Methods 3.2). Coomassie blue stained protein gels for the three DNA constructs induced in *E. coli* DLT101 are shown in Figure 44. The *E. coli* strain containing the A_{SD} construct showed a unique protein band which is not present in the control *E. coli* strain (Figure 44, A). B_{SD} containing strain showed no difference to the control when the protein band patterns were compared (Figure 44, B). The *E. coli* strain containing the A_{SD}B construct showed the presence of a strong extra protein band (Figure 44, C). This strong band was excised and further examined by two-dimensional gel electrophoresis (Figure 45). Analysis of the pattern showed that only one protein spot was present in the two dimensional gel, meaning the strong band contains only a single protein. Since the molecular weight of this extra band was different from A_{SD} gene product, which was identified earlier, it was assumed that this strong band produced from the A_{SD}B construct was the B gene product. Later it was identified that this A_{SD}B construct also produced a lower amount of A gene product (data not shown). The reasons why the B_{SD} containing strain did not produce a detectable amount of B gene product and the A_{SD}B containing strain produced an unexpected amount of B gene product will be discussed.

6.2 Production of antibodies

The BCOADc B gene product, E1 β , was produced at a high level in *E. coli* DLT101(A_{SD}B) (Figure 46). Induction conditions were optimized to obtain as much recombinant protein as possible e.g., induction at lower cell density and a longer induction period. The protein band was isolated from SDS-polyacrylamide gels and used for generation of polyclonal antibodies against E1 β protein. The antibodies were raised in rabbits by

injecting the protein as the antigen. The quantitative and qualitative assay of the generated antibodies was performed by immunoblotting the protein with a series of dilutions of the antibody (Figure 47). Total proteins from the cell lysate were separated by SDS-PAGE and transferred to immunoblotting membranes. Specific bands were obtained by incubating them with 200-fold, 500-fold, 1,000-fold and 2,000-fold diluted polyclonal antibody mixture. The 2,000-fold dilution of the antiserum was selected for Western (immunoblot) analysis of transgenic plant proteins. This dilution displayed the least background and the clearest pattern of protein (although the contaminating bands above and below the E1 β still remained visible).

Polyclonal antibodies against *luxA* and *luxB* bacterial luciferase proteins were also prepared by the same procedure. A stock of this antibody mixture (contained both anti-*luxA* and anti-*luxB*) had been used in experiments to detect *luxF* gene products in tomato plants transformed with *luxF*-B genes.

6.3 Western (immunoblot) analysis of luciferase protein in transgenic tomato plant tissues

The presence of luciferase proteins in tomato plants transformed with pPCV701-*luxFP1*-B was examined using a polyclonal anti-*luxA* and anti-*luxB* antibody mixture. In order to obtain maximum expression of luciferase fusion protein, the tomato fruit was sterilized, sliced and the *mas* promoter induced on 2,4-D containing medium plates for specific periods of time. Figure 48, C shows the detection of luciferase protein from green fruits of plants #1, #5, #10 and #11. The corresponding light units from 300 μ g of total protein from fruit tissue was measured by luminometer (Figure 48, A). Only tissue from plant #1 showed the presence of luciferase fusion protein in green fruit tissue. Similar experiments were carried out using 300 μ g total protein from red fruits, for which the slices were kept for five days on 2,4-D containing medium plates (Figure 48, B). In parallel

experiments (Figure 48, D), strong signals were obtained from plants #1, #10 and #11. Weak signal also can be seen from plant #5. Comparison of immunoblots (Figure 48, C & D) shows that protein extracts from red fruits contain higher amounts of luciferase protein than protein extracts of green fruits. However, red fruits produce less light units than green fruits from the same amount of total protein, suggesting that luciferase isolated from green tissues is more active. The reason for this activity difference is not known at present, and will be discussed.

6.4 Western (immunoblot) analysis of BCOADc gene products in transgenic tomato plant tissues

Immunoblot detection of A and/or B gene products in transformed tomato plants are illustrated in Figures 49 and 50. The *mas* promoter activity was induced for two days in leaf tissues and five days in green fruit tissues of transformed plants before immunoblot assay was carried out. A total of four independent 701-*luxF*-B transformed plants (#1, #5, #10, and #11) and five independent 701-AB transformed plants (#1, #2, #3, #5, and #6) were examined. B gene product was detected in fruit tissues (Figure 49). From five A and B gene transformed plants, only plant #1 showed protein signal at the position expected for the molecular weight of E1 β protein using anti-B antibody. Figure 50 shows the detection of the B gene products in leaf extracts in nine independent transformed plants (four transformed with pPCV701-*luxF*-B, five with pPCV701-AB). Plant #1 from 701-AB transformants showed a positive signal identical to E1 β protein position.

Figure 51 shows immunoblotting performed using total proteins isolated from *B. subtilis* 168s wide type donor strain and the CAC-11 mutant strain. The signal present in extracts from 168s (lane B1 and B2) confirms that the antibody recognizing E1 β protein detects B gene product and the position of this gene product is identical to the original

product after all the molecular engineering procedures and production in a heterologous host such as plant cells. Surprisingly, the same signal was also obtained in extracts from CAC-11 mutant cells (lane C1 and C2) which indicated that the mutation in the B gene of CAC-11 still produce E1 β polypeptide identical to E1 β wide type, meaning the *bfm* mutation is not the result of codon shift which occur by single or double nucleotide loss. Further investigation will be needed to determine the position of the *bfm* mutation in the *B. subtilis* CAC-11 strain.

6.5 Summary

The objective of detection of BCOADc and bacterial luciferase gene products was achieved by Western (immuno) blot analysis. Previous results (Results 1-5) have demonstrated that genes encoding E1 α and E1 β subunits of BCOA decarboxylase were successfully introduced into tobacco and tomato plants. Investigations also showed that genes were actively transcribed in tomato plants at different levels. Questions, such as the genes are correctly translated into the plants or the new proteins are maintained in the plant cells stably were needed to be answered experimentally. This section presented evidence that the genes are not only integrated into the plant genome, but also synthesize the encoded proteins. Overall, translational analysis was based on the immuno cross reaction procedures. The subunit proteins E1 α and E1 β were first cloned into expression vectors and overexpression was obtained *in vivo* in bacterial cells. The related proteins were then used as antigens to produce polyclonal antibodies in rabbits. The antibodies made against E1 α and E1 β proteins were then used in immunoblot assays for the detection of the translational products of BCOADc genes in plant tissues.

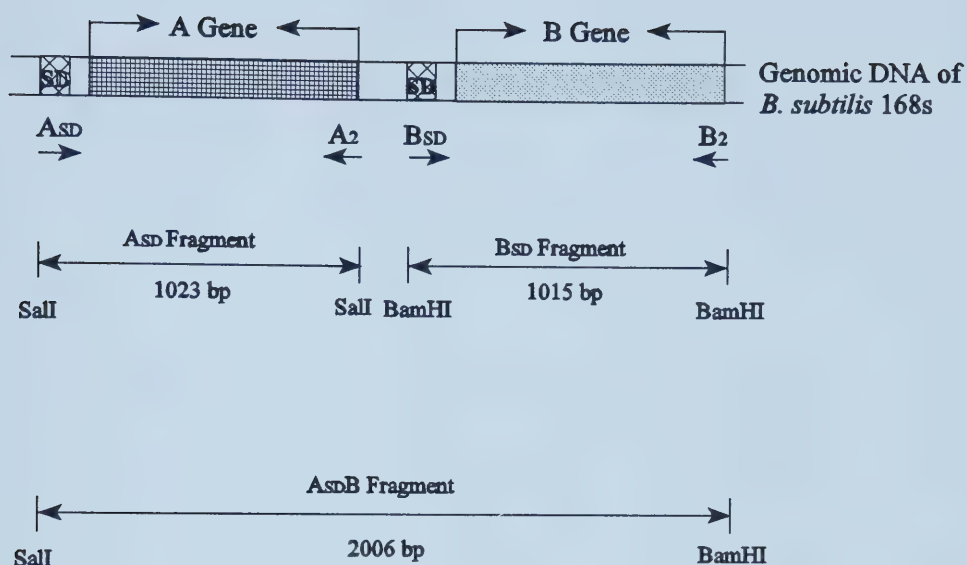


Figure 43. Excision of the A gene, B gene, and the AB genes from genomic DNA fragment of *B. subtilis* using synthetic primers and PCR followed by the addition of the required Shine-Dalgarno sequences.

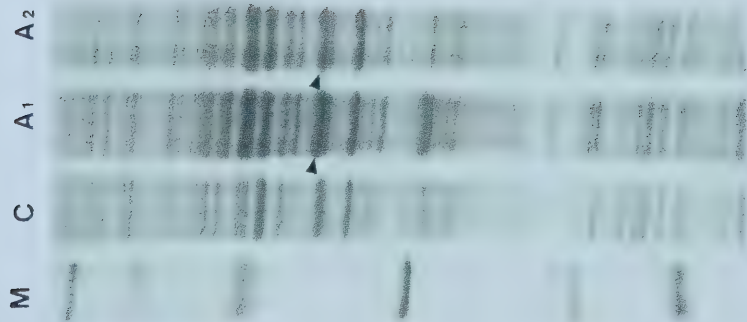
Figure 44. Overproduction of the cloned A and B gene products from the T7 promoter in *E. coli* DLT101. Total proteins from *E. coli* strains were extracted and separated on 7.5-25% polyacrylamide gradient gels.

Panel A: Expression of the A gene products. The arrow indicates the A gene product isolated from two independent subclones lane A1 and A2; lane C is the total proteins from *E. coli* DLT101 untransformed strain; lane M is the wide ranged molecular weight marker at 97 KDa, 66 KDa, 45 KDa, 31 KDa, 21 KDa, and 14.4 KDa from the top of the gel to the bottom.

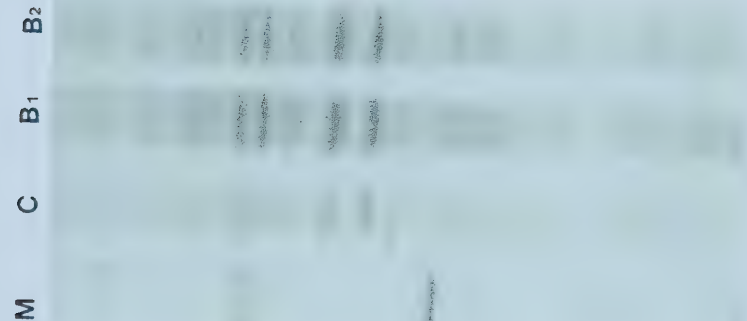
Panel B: Expression of the B gene products from two independent subclones lane B1 and B2. No extra protein band was detected; lane C is untransformed *E. coli* sample. Lane M is the wide ranged molecular weight marker at 66 KDa, 45 KDa, 31 KDa, and 21 KDa from the top of the gel to the bottom.

Panel C: Expression of the A and B genes products in the AB subunit containing strain. A single strong band indicated by arrow presents in the isolates. No identical band was present in negative control C. Lane M is the wide ranged molecular weight marker at 97 KDa, 66 KDa, 45 KDa, 31 KDa, 21 KDa, and 14.4 KDa from the top of the gel to the bottom.

A



B



C

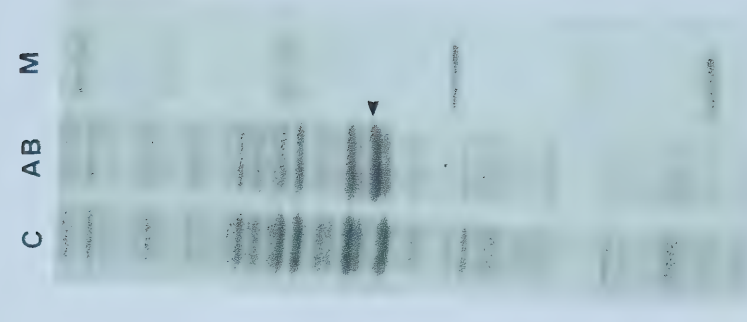


Figure 45. Analysis of the B gene product in two-dimensional (2-D) gels. Arrows indicate the B gene product in 2-D gels. Lane M is the molecular weight marker.

M



Figure 46. Optimization of induction of the T7 promoter linked B gene expression in *E. coli* DLT101.

Lane C shows the total protein isolated from 30 μ l of *E. coli* DLT101 cells at the $OD_{600}=2$; a, b, c, and d are the total proteins isolated from the B gene containing transformed *E. coli* DLT101 cells at: a, 15 μ l ($OD_{600}=1$); b, 30 μ l ($OD_{600}=1$); c, 15 μ l ($OD_{600}=2$); d, 30 μ l ($OD_{600}=2$).

M is the protein molecular weight marker at 66 KDa, 45 KDa, 31 KDa, and 21 KDa.

Total proteins were separated in 7.5-20% gradient gels by SDS-PAGE.

AB

C a b c d M

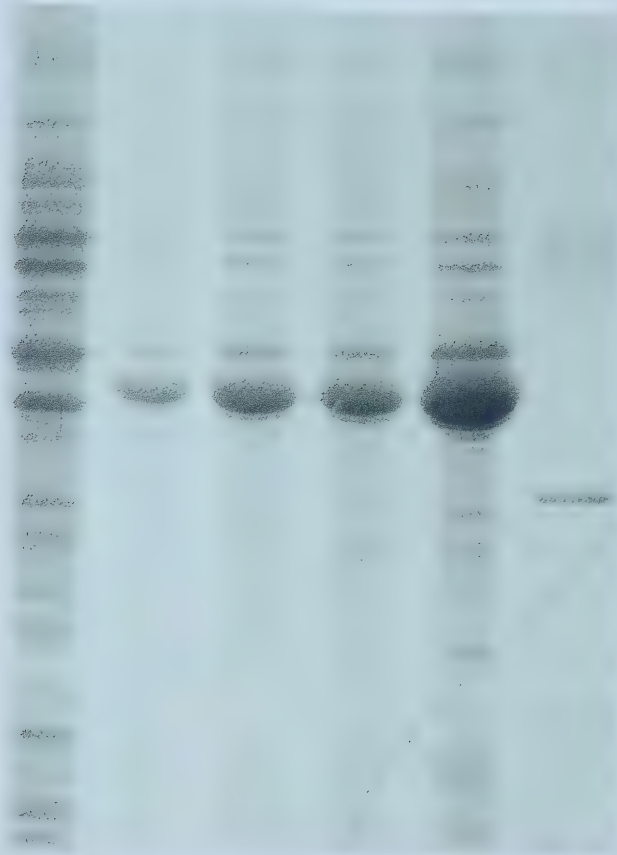
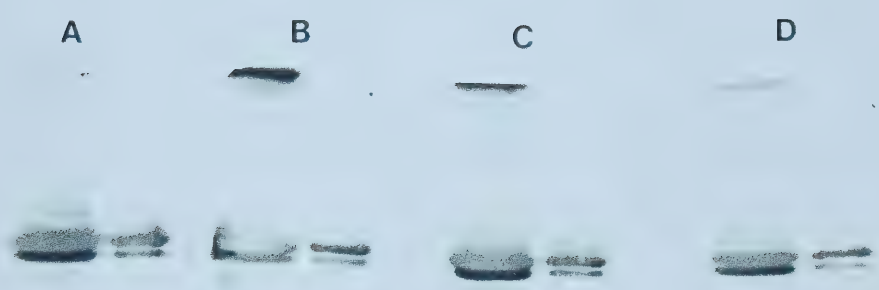


Figure 47. Determination of antiserum specificity and titer against E1 β protein using Western (immunoblot) technique.

Total proteins were isolated from the *E. coli* DLT101 strain, separated on 1 mm gradient SDS-polyacrylamide gels, and transferred to membranes.

Immunoblot using the antiserum at the titer of 1:200 (A), 1:500 (B), 1:1000 (C), and 1:2000 (D). The total proteins used for each titer were 20 μ l (left) and 10 μ l (right).



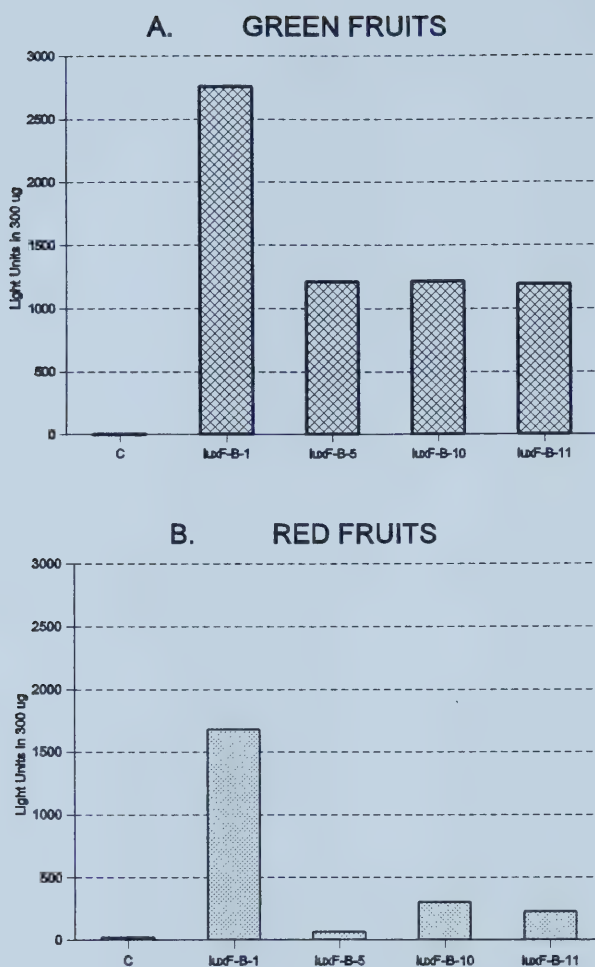


Figure 48. Identification of *luxF* gene product in protein extracts of immature fruits (green) and mature fruits (red) from four transformed tomato plants using Western (immuno) blot analysis.

Lane C is total protein isolated from untransformed tomato plants.

Lane M is the *luxF* protein synthesized in *E. coli* as a positive marker.

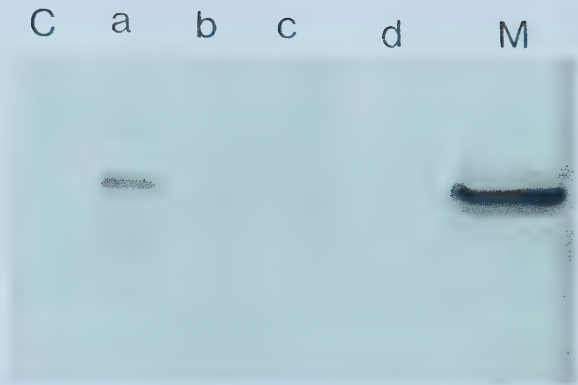
Panel A: Detection of light units from 300 µg green fruit tissues similar to those used for immunoblotting;

Panel B: Detection of light units from 300 µg red fruit tissues similar to those used for immunoblotting;

Panel C: Immunoblot using tissues from A;

Panel D: Immunoblot using tissues from B.

C.



D.

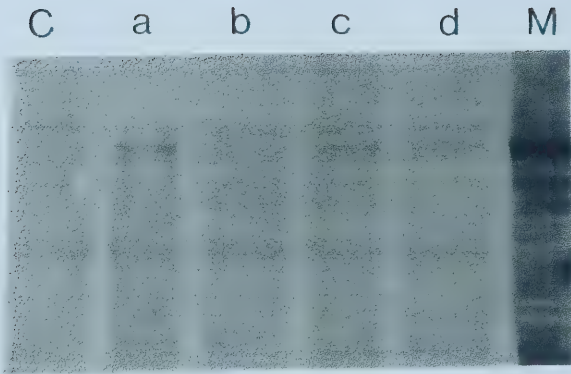


Figure 49. Western (immuno) blot analysis of BCOADc B gene product (E1 β) in extracts of green fruits of four transformed tomato plants after induction of the *mas* promoters.

Lane C is total proteins isolated from nontransformed fruit tissues;

Lane M is the total proteins isolated from *E. coli* strain containing B gene as a positive control;

Lane a is total protein isolated from fruit tissue of #1 plant transformed with *luxF*-B genes;

Lane b is total protein isolated from fruit tissue of #10 plant transformed with *luxF*-B genes;

Lane c is total protein isolated from fruit tissue of #1 plant transformed with the A and B genes;

Lane d is total protein isolated from fruit tissue of #3 plant transformed with the A and B genes.

Arrows indicate the position signal detected using anti-B antibody. The lower band indicates the detected B gene product E1 β proteins; the upper band indicates the detected a dimer of one α and one β protein subunits using anti-B antibody.

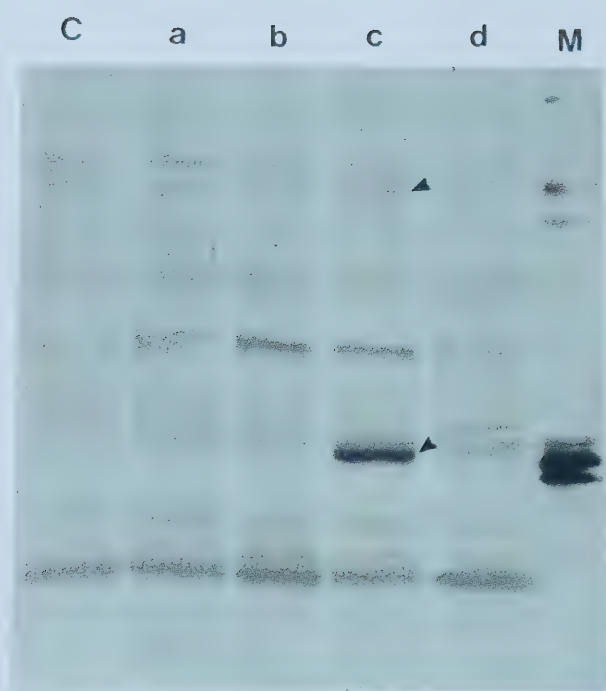


Figure 50. Immuno detection of the B gene products in leaf extracts of nine independent transformed tomato plants after induction of the *mas* promoters. Total proteins are electrophoresed in 7.5-20% SDS-polyacrylamide gels.

Lane C: Total protein from untransformed leaf tissues;

Lane a: Total protein from leaf tissue of plant #1 transformed with *luxF*-B genes;

Lane b: Total protein from leaf tissue of plant #5 transformed with *luxF*-B genes;

Lane c: Total protein from leaf tissue of plant #10 transformed with *luxF*-B genes;

Lane d: Total protein from leaf tissue of plant #11 transformed with *luxF*-B genes;

Lane e: Total protein from leaf tissue of plant #1 transformed with AB genes;

Lane f: Total protein from leaf tissue of plant #2 transformed with AB genes;

Lane g: Total protein from leaf tissue of plant #4 transformed with AB genes;

Lane h: Total protein from leaf tissue of plant #5 transformed with AB genes;

Lane i: Total protein from leaf tissue of plant #6 transformed with AB genes;

Lane j: Total protein from fruit tissue of plant #10 transformed with *luxF*-B genes;

lane k: Total protein from fruit tissue of plant #1 transformed with AB genes;

Lane l: Total protein from fruit tissue of plant #3 transformed with AB genes;

Lane M1: Total protein from *E. coli* strain containing B gene;

Lane M2: Prestained broad range molecular weight marker.

Arrows indicate the position signal detected using anti-B antibody. The lower band indicates the detected B gene product E1 β proteins; the upper band indicates the detected a dimer of one α and one β protein subunits using anti-B antibody.

C a b c d e f g h i j k l M₁ M₂

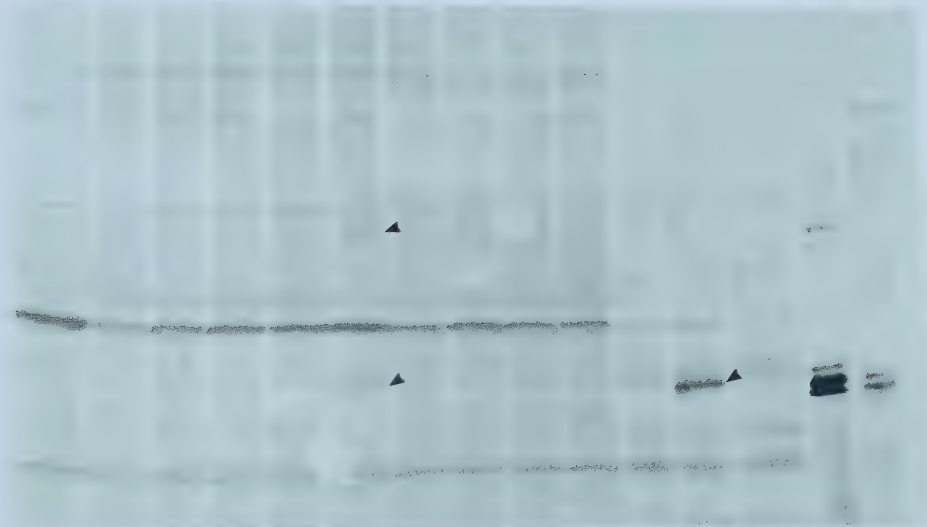
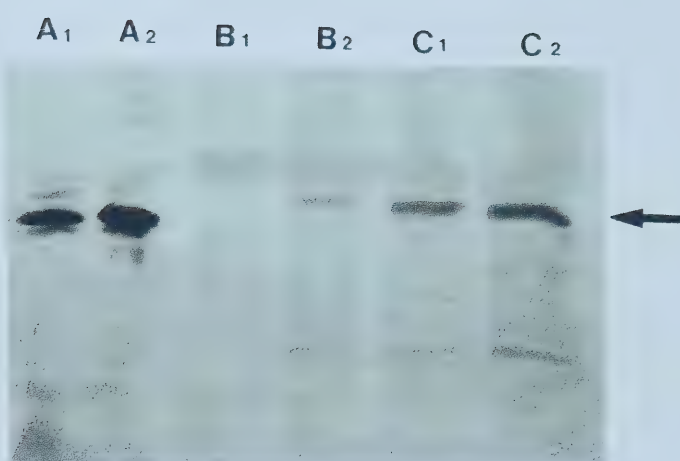


Figure 51. Western (Immuno) blot analysis of the BCOADc B gene product in extracts of *Bacillus subtilis* 168s wild type and *B. subtilis* 168s BCOADh mutant CAC-11 and in *E. coli* DLT101 transformed with the T7-AB gene construct.

Lane A1 and A2: Total proteins isolated from *E. coli* strain containing B gene from cells of 0.04 OD (A1) and 0.4 OD (A2).

Lane B1 and B2: Total proteins isolated from *B. subtilis* wide type strain from cells of 0.04 OD (B1) and 0.4 OD (B2).

Lane C1 and C2: Total proteins isolated from *B. subtilis* mutant CAC-11 strain from cells of 0.04 OD (C1) and 0.4 OD (C2).



7. Cold Tolerance of Transgenic Plants Containing BCOADc Genes

7.1 *Visual observations of cold-mediated senescence.*

Studies were carried out to determine whether tomato plants, *L. esculentum* var. Roma, transformed with genes encoding BCOADc E1 α and/or E1 β subunits can grow at 10-17°C below their optimal temperature. Seeds were obtained from the following plants characterized in earlier studies: (1) an untransformed plant, (2) five plants transformed with plasmid pPCV701-*luxFP1-B*, i.e., #1, #3, #5, #10, and #11; and (3) five plants transformed with plasmid pPCV701-AB, i.e., #1, #2, #3, #4, and #5. The seeds were sterilized and germinated in the presence of kanamycin (Methods 16.2) to produce the F1 transgenic plants for study. Approximately fifty seeds were used from each plant. After cotyledons were formed, the seedlings were transferred to jars containing MS growth medium. Seedlings without well developed root systems in kanamycin containing medium were discarded. (Some plants which do not contain the plasmid DNA are among the expected F1 offspring of the parental transgenic plant.)

After two months, true F1 plants were obtained from the pPCV701-*luxFP1-B* transformed plant #1 (referred to as B-X-1) and from the pPCV701-AB #4 transformed plant (referred to as AB-4). Untransformed plants were used as controls (referred to as C). A total of fifteen plants (3 x C, 6 x B-X-1, 6 x AB-4) were planted in soil, in a growth chamber (Figure 52) at the regular (optimal) growth temperature (22°C-18°C) (Methods 17.4). Plants were then arranged in three replicate groups, labeled I, II, and III. Each group of plants contained one untransformed plant (e.g. I-1), two B-X-1 plants (e.g. I-2 and I-5), and two AB-4 plants (e.g. I-3 and I-4). When the plants started to grow, they were moved to a different growth chamber and subjected to a controlled temperature shift regime (Table 16). Surprisingly, there were no visible changes in the appearance of the fifteen potted plants

until the temperature was lowered to 4°C - 4°C (day-night). Daily observations were recorded. After a few days incubation at this temperature, symptoms similar to senescence began to appear (for specific test plants, please see Table 17). All the leaves and the apical portion of stems became soft (lost turgor pressure), wilted and drooped (Figure 53). Wilted plants were able to recover if they were returned to the optimal growth temperature (growth chamber #2) within three days after the wilting began (Figure 54). For example, sample I-3 (transformed with BCOADc A and B genes) recovered nicely, when it was moved back to the optimal temperature growth chamber within three days after the symptoms of wilting occurred (Figure 54 A, I-3). Younger leaves recovered faster than older leaves. Some older leaves were irreversibly damaged (Figure 54 A, I-3).

After growth at 4°C for a few days, stems of all test plants started to turn dark purple in color, leaves became dark green in color with dark purple spots on the upper surfaces, but these plants were still growing albeit slowly. After 20 days at 4°C, the color of the leaves and stems of these plants turned lighter. By the 40th day, the leaves of plants which still did not show wilted symptoms had already turned yellowish in color (Figure 54). Transgenic F1 plants II-5, III-2, and III-5 (carrying only the BCOADc B gene) and transgenic plants III-3, II-4 III-4 (carrying the BCOADc A and B genes) were moved back to optimal growth conditions (22°C) after survival at 4°C for 51 days without showing wilted symptoms. Although the leaves were not able to recover, new shoots appeared from the base of the stems 1-2 weeks after the return to optimal temperature. Wilted control plants were moved back to #2 growth chamber 7 days after wilted symptoms occurred. They were not able to recover at optimal temperatures.

7.2 Luciferase activity of transgenic tomato plants in the cold

A comparison was made of *mas* promoter activation at 4°C and 22°C by measuring

the activity of the *luxF* reporter gene product, luciferase. Leaf samples were collected from the six F1 plants derived from pPCV701-*luxFP1*-B transformed plant #1 which were exposed to 4°C for seven days (see Table 16). Leaf samples were also collected from plants vegetatively propagated from these plants and maintained in the greenhouse at 22°C. Bacterial luciferase activity from extracts of the leaf samples was measured quantitatively using luminometry (Figure 55). The average luciferase activity from six plants grown at 4°C was found to be higher in both the base leaves (B) and the top leaves (T) than in plants grown in the greenhouse at 22°C, and the base leaves contained more luciferase activity than the top leaves. Each value in the chart is the average luciferase activity from six individual samples. The enhanced expression of the luciferase enzyme at low temperature suggests two possibilities which are not mutually exclusive: (1) transcription from the *mas* promoter may be enhanced by the stress of low temperature and (2) luciferase activity may be enhanced at low temperature if enzyme turnover is reduced by the cold.

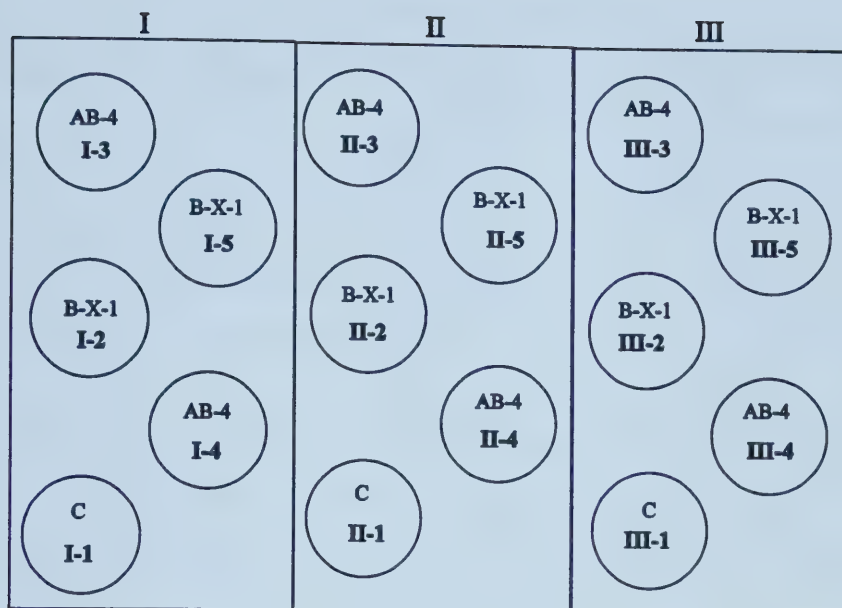


Figure 52. Organization of plants in growth chambers for temperature shift regime. A total of fifteen plants were arranged into three groups (I, II, III). Each group contained one control plant (C), two plants transformed with BCOADc A and B genes (AB-4), and two plants transformed with *luxF* and the BCOADc B gene (B-X-1). They were distributed to different locations to avoid any possible position effect in the chamber. Each plant was named according to group number (I, II, III) and given a plant number (1, 2, 3, 4, or 5).

Table 16. Temperature Shift Protocol for Testing Cold Tolerance
of Transgenic Tomato Plants

Step	Location	Temperature (Day - Night)	Date (Day-Month-Year)	Incubation Period (Days)
1	#2 Growth chamber	22°C - 18°C	06-05-94	15
2	#1 Growth chamber	16°C - 12°C	21-05-94	10
3	"	12°C - 8°C	31-05-94	15
4	"	8°C - 4°C	16-06-94	20
5	"	4°C -4°C	06-07-94	50

Table 17. Appearance of wilt symptoms in F1 tomato plants at 4°C

Plant	Appearance of Symptoms (Day-Month-Year)	Number of Days at 4°C
I-1*, I-3	11-07-94	5
III-1*	16-07-94	10
II-3	27-07-94	21
II-1 *	29-07-94	23
I-5	07-08-94	32
I-4	10-08-94	35
I-2, II-2	22-08-94	47
II-4, II-5, III-2, III-3, III-4, III-5	-	51

* Indicates untransformed plant samples.

Figure 53. Appearance of tomato plants after incubation in 4°C growth chamber.

(1) Top two rows A and B (left to right) are group I plants (I-1, I-2, I-3, I-4, and I-5).

Row A shows five plants one day after exposure to 4°C (photographed May 21, 1994).

Row B contains the same plants after exposure to 4°C for 5 days.

The wilt symptoms occurred on plant I-1 and I-3 (photographed July 11, 1994).

(2) The middle two rows C and D (left to right) are group II plants (II-1, II-2, II-3, II-4, and II-5).

Row C shows the plants one day after exposure to 4°C (photographed May 21, 1994).

Row D shows the same plants exposed to 4°C for 23 days (photographed July 30, 1994).

Plants II-1 and II-3 were wilted and drooping.

(3) The bottom two rows E and F show plant group III (III-1, III-2, III-3, III-4, and III-5).

Row E shows the plants after exposure to 4°C for one day (photographed May, 21, 1994).

Row F shows the same plants exposed to 4°C for 23 days (photographed July 30, 1994).

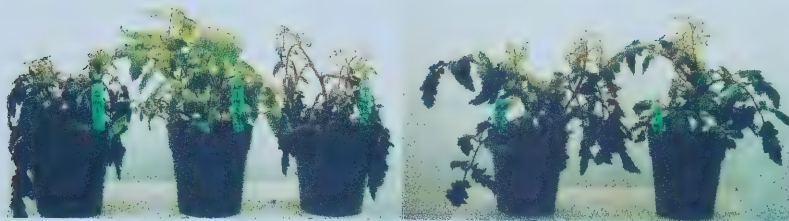
Only control plant III-1 showed the wilted symptom.

Number 1, 2, 3, 4, and 5 indicate numbers of plants in each group.

A



B



C



D



E



F



1

2

3

4

5

Figure 54. Appearance of tomato plants after prolonged incubation at 4°C. Plants were grown at 4°C for 37 days (photographed August 12, 1994). Plants from three groups are indicated as group I (row A), group II (row B), and group III (row C).

(1) The top row A, from left to right, are group I plants I-1 (control), I-2, and I-3. I-3 was recovered after being moved to #2 growth chamber (22°C).

(2) The middle row, B from left to right, are group II plants II-1 (control), II-2, II-3, II-4, and II-5. II-1 and II-3 showed wilted symptoms (turgor pressure was lost from these plants).

(3) The bottom row C, from left to right, are group III plants, III-1 (control), III-2, III-3, III-4, and III-5. Only III-1 plant showed wilted symptoms after 37 days.

A



B



1

2

3

4

5

#1 (pPCV701-luxF-B)

at 4 degree and 22 degree C

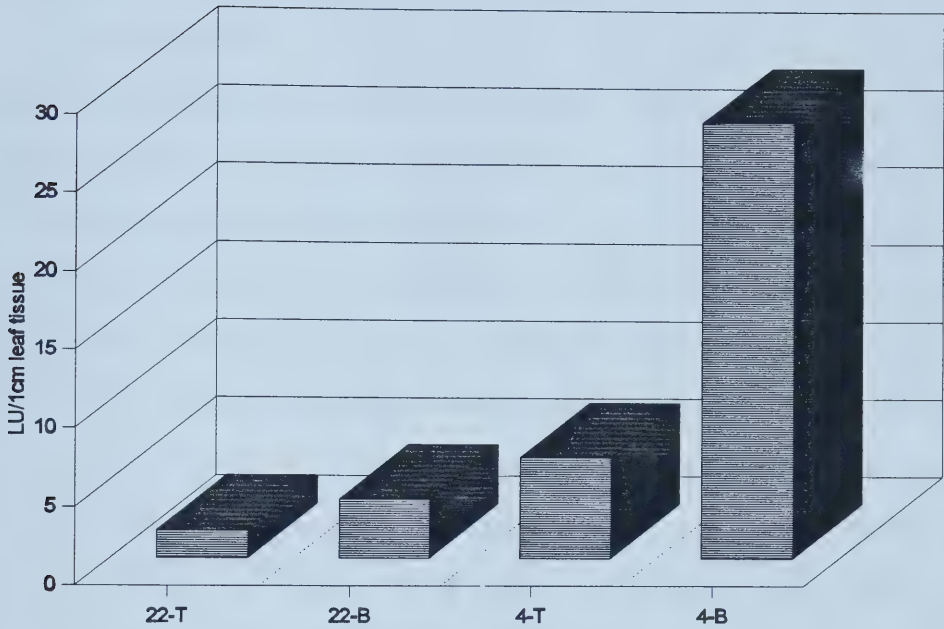


Figure 55. Expression of luciferase gene driven by *mas* promoter in leaf tissues of plant #1 transformed with pPCV701-luxFP1-B gene cassette.

22-T sample is a leaf disc ($\phi 1\text{cm}$) from the top of plant #1 grown in greenhouse (22°C);

22-B sample is a leaf disc ($\phi 1\text{cm}$) from the bottom of plant #1 grown in greenhouse (22°C);

4-T sample is a leaf disc ($\phi 1\text{cm}$) from the top of plant #1 grown in the growth chamber (4°C);

4-B sample is a leaf disc ($\phi 1\text{cm}$) from the bottom of plant #1 grown in growth chamber (4°C).

DISCUSSION

1. Cloning and DNA sequencing of BCOADc genes

DNA sequencing of the 3,700 base pairs genomic DNA from *B. subtilis* 168s demonstrated that there were three open reading frames, which are homologous to the E1 α , E1 β and E2 components of reported α -oxo acid dehydrogenase complexes (Table 9). This result, combined with the fact that the *bfmB* mutation causes the loss of the branched-chain α -oxo acid dehydrogenase activity, indicated that these open reading frames were E1 α , E1 β and E2 genes of the branched-chain α -oxo acid dehydrogenase complex, respectively. Following the stop codon of the E2 gene was a putative transcription termination site with inverted repeats (Figure 11). A similar transcription termination site after the E2 gene was found in the case of the 2-oxoglutarate dehydrogenase complex of *B. subtilis* (Carlsson and Hederstedt, 1989). In both cases, the E3 gene was not found in the location adjacent to the E1 and E2 genes. Thus, the lipoamide dehydrogenase (E3) gene of the pyruvate dehydrogenase complex was probably shared by the three dehydrogenase complexes: pyruvate, 2-oxoglutarate and branched-chain α -oxo acid.

Based on the information of genomic mapping, the *aceA* gene is located on the circular genetic map of *B. subtilis* at 126° and encodes the E1 α component of the pyruvate decarboxylase complex (Boudreaus et al., 1981; Hemila et al., 1990). The E1 α , E1 β and E2 operon of the branched-chain α -oxo acid dehydrogenase complex, as shown in this paper, is positioned at 212° (Boudreaus et al., 1981). Recently, two α -oxo acid decarboxylases, namely pyruvate and branched-chain α -oxo acid, have been isolated from *B. subtilis* and their physical and catalytic properties characterized in detail (Oka and Kaneda, 1988). Immune-precipitation experiments have clearly shown that the branched-chain α -oxo acid decarboxylase (E1 α & E1 β), but not pyruvate decarboxylase, is essential for the

incorporation of branched-chain α -oxo acids into branched-chain fatty acids. The apparent dissociation of the branched-chain α -oxo acid decarboxylase from the branched-chain α -oxo dehydrogenase complex present in *B. subtilis* was not an artifact of protein purification.

Primary structure of the three open reading frames suggest that they belong to one operon. Therefore, one transcript was originally produced in the bacterium. The recognition of ribosomal binding sequences in front of each open reading frame also supports this hypothesis. However, it was difficult to identify promoter sequences on the basis of comparisons with known *Bacillus subtilis* promoters. It will be necessary to conduct primer extension experiments or S1 nuclease mapping to determine precisely the site at which transcription is initiated and, by inference, the location of the promoter sequences.

2. Benefit of automated sequencing

The Applied Biosystems DNA Sequencer is an automated gel electrophoresis detection system. It separates, detects, and identifies fluorescently labeled DNA molecules such as the products from dideoxy sequencing reactions. The system is composed of (1) an electrophoresis/detection unit, (2) a computer that includes software for data display and interpretation, (3) a plotter, and (4) a printer. Dideoxy sequencing reactions are performed with subpicomole quantities of fluorescent dye-terminators (2', 3'-dideoxyribonucleoside 5'-triphosphates) instead of the usual radioactive labels. All commonly used polymerases can be used for primer extension. The most popular choice is T7 DNA polymerase. Just recently, ABI has developed another independent dye-terminator system to recognize sequencing products catalyzed by sequenase. In both cases, four different fluorescent dyes distinguish the products from each of the four sequencing reactions. These reactions are combined and loaded into one lane (instead of four) on the gel. Up to 36 clones (templates) can be sequenced simultaneously on one gel. The four dyes are detected by laser-stimulated

fluorescence and the information stored by the computer. Ten to 12 hours are usually required for separation and detection of 350 to 450 bases.

The ABI automatic sequencer facilitated the completion of the sequencing of 3,700 bp at genomic DNA of *B. subtilis* 168s. There were five compression regions in the three open reading frames that consisted of 3-5 "G" (guanosine) and 1-2 "C" (cytidine). The exact sequences of these nucleotides could not be determined on sequencing gels by manual sequencing due to the incorrect spacing. By thermal cycling of the reactions carried out during automated sequencing, the strong secondary structures of these compression regions were completely destroyed, so that the primers could correctly copy the DNA sequence (Figure 56). Nucleotide sequences at the location of 182-187 indicated a highly compressed region consisting of the six nucleotides 5'-GGGGCG-3'. The fluorescent signals correctly reflected the actual sequences when automated sequencing reactions and signal detection systems were employed.

3. Transfer of the A and B genes of BCOADc into plants

Based on DNA sequencing data, the structural genes A and B (ORF1 and ORF2) encoding E1 α and β subunits of BCOADc were isolated by PCR. The polymerase chain reactions allowed the subcloning of E1 α and E1 β encoding genes A and B into the plant expression vectors. Transgenic tobacco and tomato plants were successfully constructed using these vectors, in which the A gene was under the control of the *mas* P1 promoter and the B gene was under the control of the *mas* P2 promoter. The confirmation of correct DNA sequences for both genes eliminated the possibility that mutations were introduced by PCR during DNA synthesis. Thus, the correct expression of E1 α and E1 β encoding genes in bacteria and in plants should be possible. The following subcloning processes were achieved by introducing the A and B genes into plant expression vectors. Plasmid pPCV701-AB was

generated by subcloning the A and B genes into pPCV701 vector driven by P1 and P2 promoters (P1A and P2B), respectively. Plasmid pPCV701-*luxFP1-B* was created by introducing the B gene of BCOADc into the pPCV701-*luxFP1* vector downstream of P2 promoter. These two expression vector systems were then used to transfer tobacco (SR1) and tomato (Roma) plants. The transformation of tobacco and tomato plants was achieved using *Agrobacterium* mediated explant transformation procedures. Genetic (Southern hybridization) and biochemical analyses (bioluminescent assay) verified that T-DNA carrying the P1A and P2B gene cassette or carrying the P1*luxF* and P2B gene cassette has been successfully integrated into the genome of tobacco and tomato plants; regenerated, transformed plants of both species have been obtained and maintained.

The transcription of the A and B genes regulated by the *mas* promoter was analyzed by primer extension and RNase protection assays. (Primer extension procedures turned out to be more sensitive than Northern hybridization for these particular studies.) The results from primer extension reflect the levels of the transcriptional activity. All the transformed tomato plants tested showed transcriptional levels consistent with different levels of gene expression.

The presence of the translation products the E1 α and E1 β polypeptides in the transgenic plant tissues was demonstrated by immunoblot analysis.

4. Transformation

Agrobacterium introduces one to several copies of the transferred DNA into the plant genome at one or a few loci. The site of insertion is not restricted to any particular chromosome or site on a chromosome. These features may make *Agrobacterium* a vehicle for insertional mutagenesis. Upon introduction of foreign genes into plant genomes, the normal activity of genomic DNA may be destroyed by activating or inactivating genes

adjacent to the inserts. Such disorders are usually apparent in the phenotype of the transgenic plants. In fact, one tomato-AB transformed plant (#3) has shown a severe reduction in production of fruits and this plant also produced fewer flowers than others. Most of these flowers were aborted during development. When fruits were produced, the size of these fruits was much smaller. When the seeds were investigated, it was found that only very few seeds were produced and most fruits did not produce any seeds. These disorders, therefore, appear to affect hormone metabolism or maturation processes. Reduced seed production from transgenic plants have been reported earlier. Similar phenomena were also found in several transformed plants containing the BCOADc AB gene constructs or the *luxF*-B construct.

During the *Agrobacterium* mediated explant transformation processes, it was found that leaf discs transformed by *Agrobacterium* containing the pPCV701-*luxFP1*-B construct always generated more shoots than the other constructs (pPCV701-AB and pPCV701-*luxA* & B) both in tobacco and tomato plants. The reasons for this are unknown. It is possible that the plasmid carried by the *Agrobacterium* strain may have altered plant hormone biosynthesis and activity in a manner which led to the shoot formation process. It is important to note, however, that although this construct produced more shoots, these shoots gave rise to a higher ratio of untransformed plants than with the other constructs. Transformed plants were selected by the expression of a selectable marker gene, the neomycin phosphotransferase (NPTII) gene, which confers resistance to kanamycin. The strength of the selection depended upon the concentration of the antibiotic kanamycin provided in the growth medium. Transformants were selected in this study from *Agrobacterium*-treated tobacco and tomato explants using kanamycin at the concentration of 100 mg per liter of medium. Of the 36 plants, which were regenerated from transformations with three different constructs, nine plants (i.e., 25%) were considered to be untransformed (Table 12, 14 and 15; Figures 22 and 26). Of these nine plants, six (67%)

were regenerated from pPCV701-*luxFP1-B* transformation (as described above). This may be due to the ability of leaf discs transformed with pPCV701-*luxFP1-B* to generate more spontaneous kanamycin resistant shoots.

5. T-DNA can integrate into the plant genome more than once

Foreign DNA inserts were analyzed using several independently transformed plants. Recent publications have indicated that the transgene copy number can be positively or negatively associated with transgene expression (Hobbs et al., 1993; Grevelding et al., 1993). In most cases, single segregation loci have been observed, followed by linked and unlinked multiple loci. In addition, multiple T-DNA insertions have been commonly observed. They are frequently organized as direct repeats in head-to-tail arrays (e.g., RB/LB), as inverted repeats in head-to-head arrays (RB/RB or LB/LB inverted repeat structure), or in even more complex structures. This project demonstrated that T-DNA was inserted into plant genomes by single insertion at one position or several positions, or multiple insertions at one position or several positions. For example, tomato plants #1 and #4 transformed with pPCV701-AB construct and tomato plants #1 transformed with pPCV701-*luxFP1-B* construct were carried a single insert. Tomato plants #2, #3, #5 and #6 transformed with the pPCV701-AB construct and tomato plants #3, #5 and #10 transformed with the pPCV701-*luxFP1-B* construct contained multiple inserts. Tomato plant #2 transformed with the pPCV701-AB was unique in that it carried multiple copies of the A and B genes at a single insertion site in a head-to-tail array. Variations in the expression levels of these inserts, due to copy number, will be discussed later.

6. Transcriptional analysis of BCOADc genes in transgenic plants

The relationship between gene copy number and transcription level differed in tomato

plants transformed with the AB gene cassette as compared to the *luxF*-B gene cassette. Among the transgenic tomato plants containing the AB gene cassette, plant #1 with a single copy of these genes exhibited the highest level of transcription while plants #3 and #5 with multiple copies of these genes exhibited lower levels of transcription. In contrast, among the transgenic plants containing the *luxF*-B gene cassette, plant #1 with a single copy of the B gene exhibited a lower level of transcription, while plant #10 with multiple copies of this gene exhibited a higher level of transcription.

It seems likely that the difference in transcription levels may be caused by position effects, since T-DNA does integrate randomly into the plant genomes. *In situ* hybridization methods can be applied to locate the gene on a specific chromosome in the genome and to determine the position of the genes on a chromosome. It is well understood that DNA with euchromatin structure is actively transcribed, whereas DNA located in the heterochromatin regions is mostly silent. Recent data suggest that genes within the heterochromatin structure can also be transcribed at very low levels in a mammalian chromosome (Wang & Szalay, unpublished data). Multiple copies of genes inserted into the plant genome do not seem to produce higher levels of transcriptional products when compared to a single copy of a gene.

Previous studies have indicated that the dual *mas* promoter exhibits slightly different transcriptional activity from the P1 and P2 promoter regions (Langridge and Szalay, personal communication). The results of primer extension studies of transcriptional levels in transgenic plants containing a single copy or a multiple copy of BCOADc genes differed (Figure 31). The B gene under the control of the P2 promoter produced more transcripts than the A gene under the control of P1 promoter in transgenic tomato plant #1 carrying a single copy of the A and B genes. However, the production of A and B transcripts in transgenic plants #3 and #5 containing multiple copies was not only significantly reduced compared to plant #1, but also appeared to be equally regulated. Analysis of banding

patterns from Southern (genomic) hybridization studies suggested that plant #5 carried the multiple copies as head-to-tail arrays and tail-to-tail arrays (two strong bands in Figure 28, A). In contrast, transgenic plant #10 carrying multiple copies of *luxF-B* produced more B gene transcripts than plant #1 carrying a single copy of *luxF-B* (Figure 28, B). Because the multiple copies of these genes in plant #10 are dispersed, rather than in the arrays described above, it appears that multiple genes organized in array structures in plant genomes may exhibit the least efficient gene transcription.

7. Complex relationship between gene copy number, method of promoter induction, and production of luciferase

Analysis of luciferase activity in transgenic tomato plants #5 and #10 carrying multiple copies of *luxF-B* showed that both #5 and #10 plants produced a higher level of luciferase enzyme activity than transgenic tomato plant #1 carrying a single copy of *luxF-B* under both uninduced and wounding-induced conditions. In contrast, under auxin-induced conditions, both the green (immature) and red (mature) fruits of plant #1 produced up to 10 times higher luciferase activity than plants #5 and #10 (Figure 48). Comparison of luciferase activity following wound-induction and auxin-induction suggests differential activation of the *mas* promoters. Auxin induction of *mas* promoters in plant #1 was the highest of all transformed plants tested (Results 6). One possibility for differential activation could be multiple regulatory sites in the promoter region which respond to the different activators. Such a high luciferase activity was not seen in plant #1 under non-induced and wound-induced conditions at both 22°C and 4°C (Figures 40-42). Turning on vegetative growth or inhibiting senescence due to the integration of T-DNA (containing *luxF-B*) may explain the high luciferase activity in plant #1 under auxin-induced conditions. If this is true, cells in this plant would be expected to be more vegetatively active; but observations of this plant

throughout development showed no enhancement of vegetative growth.

In addition, there are two alternative explanations for the observation that the auxin-induced luciferase activity of plant #1 not only reached a higher level but also continued to produce for a longer period of time than the activity measured in plants #5 and #10 (Figure 48). First, the pool of amino acids used for luciferase production may be limited. If this were true, plant #1 would be expected to require less exogenous auxin to induce its single copy of the *mas* promoter-*luxF-B* insert than plants #5 and #10 which carry multiple copies of this insert. This would result in a higher level of luciferase activity and longer duration of luciferase production in plant #1. Second, the greater auxin requirement for induction of *mas* promoters in transgenic plants with multiple copy inserts of *luxF-B* may force these plants to go from vegetative growth into senescence, during which rapid protein degradation occurs. This would lead to a lower net luciferase activity (synthesis less degradation). This explanation is also consistent with plant #10 continuing to produce luciferase for a shorter period of time than plant #1.

Wounding-induction of the *mas* promoter was also analyzed at 22°C and 4°C. Bacterial luciferase gene expression in tomato fruit tissues was used to assess *mas* promoter induction by wounding. The activation levels differed at these temperatures. The *mas* promoter system was induced to its highest levels in 48 hours at 22°C, then declined, whereas *mas* promoter activity reached this level after four days incubation at 4°C. From a total of three plants, plant #10 exhibited the highest *mas* promoter activity and plant #1 displayed the lowest *mas* promoter activity after wounding induction. The temperature effect could be explained by the following hypothesis. During wounding induction at 22°C, either a repressor for the *mas* promoters is removed or an activator for the *mas* promoters is produced. During wounding induction at 4°C, transcription from the *mas* promoters is lower (than at 22°C), thus producing less luciferase. However, less degradation at the lower

temperature leads to an increase (accumulation) in luciferase activity for four days.

Previous studies suggest a relationship between wounding and plant hormone levels. Wounding *Nicotiana tabacum* leaves causes a decline in endogenous indole-3-acetic acid (Thornburg, et al., 1991). It was also found that wounding induces the production of anionic peroxidase in green tomato fruits (Sherf, et al., 1993). An anionic peroxidase purified from tomato fruits has previously been demonstrated to have significant ascorbate peroxidase activity and indole-3-acetic acid (IAA) oxidase activity *in vitro* (Brooks, 1986), and has been proposed to function *in vivo* as an IAA oxidase (Kokkinakis and Brooks, 1979). Wounding also induces protein inhibitor I and II systems in tomato, potato and tobacco plants (Johnson, et al., 1990; Sanchez-Serrano, et al., 1987; Thornburg, et al., 1987). Thornburg et al. (1990) showed that the levels of IAA in bulk tissues decline by two to threefold after wounding, therefore, causing a derepression of the proteinase inhibitor II system. In addition, wounding has been reported to increase ethylene production (Herner and Sink, 1973); however, ethylene production or ethylene biosynthesis inhibitors do not appear to affect *mas* promoter activation in leaf tissues (Langridge & Szalay, unpublished data).

These observations suggest a model for the evolution of different regulatory sites in the *mas* promoters to allow induction by both wounding and plant hormones. When T-DNA becomes integrated into plant genomes, the mannopine synthase gene on the T-DNA may have a regulatory site activated by the wound-inducible proteinase inhibitor systems. However, auxin levels decline following wounding and auxin is required for the production of opines which the bacterium uses as food. To compensate for this reduction in endogenous plant auxin, *Agrobacterium* T-DNA contains two genes whose products cause the plant to overproduce auxin. In addition, because increased auxin levels turn off the proteinase inhibitor systems, *mas* promoter activity can no longer be activated by this wounding-induced system. In order to continue to produce opines (food for *Agrobacterium*), auxin-

induced activation at a different regulatory site in the *mas* promoters may have also evolved. Therefore, *mas* promoter induction by both wounding and auxin benefits *Agrobacterium* during its genetic colonization.

8. Luciferase

Genetic constructs expressing the *lux* reporter gene under the control of the dual *mas* promoter facilitated the identification of transformed plants. Independently transformed plants showed variable levels of gene expression (Table 6.1, 6.2, 6.3, 6.4). Because the P1 and P2 promoters are similarly regulated and of almost equal strength, the high level expression of luciferase protein encoded by the *lux* reporter gene under the control of one *mas* promoter can be used to estimate the expression of a foreign gene under the control of the other *mas* promoter. Thus, the use of the *luxF*-B construct allows the early identification of transformed plants from their luciferase activity. Southern (genomic) analysis can then be used to confirm the integration of the BCOADc B gene.

The following example illustrates the estimation of foreign gene expression using the *lux* reporter gene. Total soluble proteins were isolated from ripened red tomato fruits of plants #1 and #10 transformed with the *luxF*-B construct. Bacterial luciferase activity was determined by luminometry and the amount of luciferase protein was quantified by Western (immuno) blot analysis (Table 18). Calculations based upon these measurements showed that transgenic plant #1 produced approximately 13.2 ng luciferase protein/g ripened fruit tissue; transgenic plant #10 produced approximately 26.9 ng luciferase protein/g ripened tissue. (These levels correspond to 27.1 ng luciferase protein/mg soluble protein in plant #1 and 55.2 ng luciferase protein/mg soluble protein in plant #10.) Luciferase expression from the *mas* promoter in red ripened fruit is probably due to its naturally high level of auxin. The two-fold difference between luciferase expression in plants #1 and #10 may be due to the

observed difference in copy number of the *luxF*-B insert. In contrast, Delannay et al. (1989) have estimated that expression of the *B. thuringiensis* insecticidal toxin protein under the control of the dual *man* promoter in tomato plants was only about 1 ng protein/mg of soluble protein.

Table 18. Production of bacterial luciferase fusion enzyme
in mature fruits of transformed tomato plants

Sample	Sample weight (mg)	Light Unit (Peak)	Light Unit/g tissue	ng luciferase/g tissue (ng)
#1	238.4	55.1	264.1	13.2*
#10	181.0	97.3	537.6	26.9*

* Both data were calculated based on the following facts. It was calculated that 1 gram mature tomato fruit var. Roma contained 487 µg soluble proteins. Evaluation of luciferase protein from immunoblotting was that 150 µg soluble proteins produced 200 Light Units contained 10 ng luciferase proteins.

Genetic constructs expressing the *lux* reporter gene under the control of the dual *mas* promoter also facilitated the estimation of gene expression levels in plant tissues at different temperatures. Luciferase activity in leaf tissues of transformed plant #1 grown at 4°C was higher than in leaf tissues of the same plant grown at the optimal temperature of 22°C (Chapter 10.2). There are several possible explanations for this phenomenon. First, the half-life of luciferase protein at 4°C may be longer than at 22°C due to less degradation at the lower temperature. Second, the folding efficiency of luciferase protein at 4°C is likely to be greater than at 22°C. And third, the low temperature may enhance the basal level of transcription from the dual *mas* promoter. Transgenic plants will need to express the

BCOADc A and B gene products at a high level in order to synthesize branched-chain fatty acids for increased membrane fluidity at low temperatures. Therefore, it is fortuitous that gene expression under the control of the *mas* promoter occurs at such a high level at low temperature.

9. Detection of BCOADc encoded E1 α and E1 β protein subunits in bacteria and in plants

Of five transgenic plants transformed with the AB construct, Western (immuno) blot analysis detected E1 β polypeptide in plant #1 (Figure 50). Of four transgenic plants transformed with the *luxF*-B construct, the same method failed to detect the E1 β polypeptide (Figure 50) but successfully detected the luciferase protein in all four plants (Figure 48). An explanation for this difference is suggested by observations made during the expression of the A and B genes in *E. coli* (Figure 44). When the A and B genes are expressed in *E. coli* separately, the expression level is lower than when they are expressed together in the same host. Production of the A gene product, but not the B gene product, can be visualized by SDS-PAGE when the genes are expressed in different *E. coli* hosts. Both A and B gene products can be visualized, when the genes are expressed in the same host. A similar phenomenon was also observed in expression of bacterial luciferase A and B genes in *E. coli* (Baldwin, 1984). In this case, when the B gene of luciferase is present, the A gene product is detected at a higher level than when the B gene is absent. Thus, in both examples, one gene product appears to influence the stability of the other gene product. This would explain why the E1 β polypeptide may not be able to accumulate to detectable levels in plants transformed with the *luxF*-B construct. This apparent requirement for A gene product (E1 α) to stabilize B gene product (E1 β) in order to detect B gene expression by Western (immuno) blot analysis suggests that whenever B gene product is detected in fruit and leaf tissues of

a transgenic plant containing the AB construct, the A gene product must also be expressed (Figure 49 and 50).

Differences in the expression of eukaryotic and prokaryotic genes raise the question of whether the BCOADc E1 α and E1 β subunits will be able to assemble normally in plant cells. In prokaryotes, an operon is the major transcriptional unit. A typical operon includes a promoter sequence at the 5' end of the DNA, more than one structural gene, and a palindromic sequence which acts as a transcriptional terminator downstream of structural genes. Each structural gene has its own ribosome binding site upstream of its translational start codon (ATG). The translation of the *B. subtilis* E1 α and E1 β subunits from the same mRNA transcript (Figure 11) may facilitate their assembly in bacterial cells. Because the expression of the BCOADc A and B genes is from different transcripts (under control of dual *mas* P1 and P2 promoters) in plants, it was not known whether these subunits would be able to assemble into a functional heterodimer. The deduction that E1 α must be present to permit sufficient accumulation of E1 β for detection on SDS-PAGE gels suggests that E1 α and E1 β are able to assemble into a heterodimer in plant cells. Denatured polyacrylamide gel electrophoresis and Western (immuno) blot analysis has shown the presence of a polypeptide dimer which contains the E1 β polypeptide (Figure 50); whether the dimer also contains the E1 α polypeptide has yet to be demonstrated. Furthermore, using the same pPCV701 expression vector, Koncz et al. (1987) have reported that the α and β subunits of the bacterial luciferase enzyme are properly assembled in plant cells (Koncz et al., 1987).

Another difference between bacterial cells and plants, which may affect the successful production of branched-chain fatty acids is the compartmentalization of biosynthetic activities in plants. It is not known for certain whether the *B. subtilis* BCOADc will need to be compartmentalized with the majority of straight-chain fatty acid enzymes in the chloroplasts of leaves or the proplastids of fruit and other tissues. If the bacterial enzyme

must enter the chloroplast to catalyze the production of branched-chain fatty acid precursors, then there may be a requirement for fusing a chloroplast-leading sequence to the BCOADc A and B gene sequences. Because most of the enzymes involved in fatty acid biosynthesis are encoded in the nucleus instead of the chloroplast, it was thought for many years that a chloroplast-leader sequence was required for their compartmentalization in this organelle. More recently, however, the entry of the Rubisco polypeptide small subunit in chloroplasts has been shown to not require a chloroplast-leader sequence. For this reason, it was not considered necessary to fuse chloroplast-leader sequences to the genes encoding the E1 α and E1 β subunit polypeptides. The issue of whether the BCOADc is compartmentalized could be resolved in a future experiment. Western (immuno) blot analysis of chloroplast and proplastid total proteins probed with antisera recognizing the BCOADc gene products will answer this question.

10. Luciferase protein concentration and luciferase activity in plant tissues

Translational analysis of the bacterial luciferase fusion (*luxF* gene) in tomato revealed that auxin induction of the *luxF*-B construct in green (immature) fruit produced a higher luciferase activity/mg total protein than in the red (mature) fruit (Figure 48 A and B). However, quantitation of the amount of luciferase protein/mg total protein in these same tissues revealed that less luciferase protein was present in the green fruit than in the red fruit.

The relative levels of luciferase activity in immature and mature tomato fruits can be explained by the fruit ripening process in ripe tissues. Cell membranes of fruit tissues are broken down, resulting in the release of free fatty acids (RCOOH) which can be converted to long-chain aldehydes (RCHO). RCHO (up to 14 carbons) can be used by bacterial luciferase to oxidize reduced FMN and simultaneously emit light at 490 nm (Chen et al., 1989). The red tomato tissue produces its own substrate so that it does not require decanal.

Since oxidized FMN is not released quickly from the enzyme complex, the bacterial luciferase would not be readily available for further oxidation of FMN when assayed *in vitro*. This could explain the lower levels of luciferase activity in mature fruits, in spite of higher amounts of protein.

The relative levels of luciferase protein in immature and mature tomato fruits can be explained by differences in plant hormone levels during fruit maturation. In mature fruit tissues, the higher endogenous levels of auxin should mediate a stronger activation of the dual *mas* promoter than the lower endogenous level of auxin in immature fruit tissues. This increased level of transcription should lead to an increased level of luciferase protein production in mature fruit tissues.

In green fruit tissues, however, since the ripening process (membrane degradation) does not occur, no luciferase catalyzed reactions can take place under natural conditions. Thus, under induced conditions (wounding or wounding plus auxin), the newly synthesized luciferase accumulates. These active enzymes catalyze the luciferase reaction *in vitro* after addition of decanal which results in the emission of strong light (Figure 48, A). However, the total amount of luciferase protein in red fruit tissues is higher than in green tissues. This explains why lower luciferase light units resulted in stronger immuno blot signals in red fruit, whereas weaker protein signals were obtained from green tissues (Figure 48, C & D). In conclusion, it should be noted that when bacterial luciferase genes are used as reporter genes to monitor promoter activities for comparisons, it is important to choose biological material (bacterial, plant or others) at similar stages of development.

11. Plant tolerance to cold

When the plant cells are exposed to temperatures near 0°C, viability declines (Rao et al., 1977). Activities of overall photosynthesis, photosynthetic electrontransport, and

phosphorylation all diminish (Forrest et al., 1957; Jansz and MacLean, 1973; Rao et al., 1977; Ono and Murata, 1981a; Vigh and Joó, 1983; Vigh et al., 1985). Also, potassium ions, glutamate, and pteridines are released from the cells (Forrest et al., 1957; Jansz and MacLean, 1973; Ono and Murata, 1981b; Vigh et al., 1985), and morphological alterations of the plasma and the thylakoid membranes are detectable (Brand et al., 1979). These low temperature-dependent phenomena are irreversible, and even when the cells are again warmed to growth temperature, only partial recoveries or none at all were observed. The critical temperatures for irreversible damage of the cells depends on the growth temperature (Rao et al., 1977; Ono and Murata, 1981a, b; Vigh et al., 1985), suggesting that the temperature-dependent alteration of membrane lipids (Holton et al., 1964; Sato et al., 1979) may be involved in the susceptibility to low temperature.

A mechanism has been proposed for this low temperature-induced irreversible phenomena. The plasma membrane and thylakoid membranes are barriers for ions and small molecules, whereas the outer membrane of the cell envelope is permeable to them. At the optimal growth temperature, the plasma membrane and thylakoid membranes are both in the liquid crystalline state and are impermeable to ions and small molecules. With a decrease in temperature, the thylakoid membranes go into the phase separation state and become permeable to ions and small molecules. Under these conditions, physiological activities such as photosynthesis and photosynthetic ATP formation are reversibly diminished (Murata et al., 1975, 1983; Ono and Murata, 1979). With a further decrease in temperature, the plasma membrane enters the phase separation state and becomes permeable. Under these conditions, ions and small molecules in the cytoplasm leak out, and those in the surrounding medium leak in. This diminishes cellular metabolism leading to the death of the cell. Even when the temperature is raised to that which supports growth in unchilled cells, the concentrations of ions and small molecules are not recovered, thus resulting in irreversible damage of all

physiological activities of the cells. Thus, a phase transition in the plasma membrane is directly related to low-temperature damage.

The effects of E1 α and E1 β polypeptides and E1 β polypeptide alone in transformed plants were tested by comparative analysis of transformed and untransformed (control) tomato plants subjected to a cold temperature regime. Among 15 plants tested at 4°C, three untransformed plants showed symptoms of damage much earlier (at the fifth, tenth and 23rd day of exposure) than plants transformed by genes encoding E1 α and E1 β subunits of BCOADc. This suggests that plants containing and expressing the BCOADc A and B genes or only the B gene seem to be better able to survive in the 4°C environment. This result also suggests that the B gene product may be able to catalyze the required step for branched-chain fatty acid synthesis in the absence of the A gene product in plants. This is not surprising since the B gene product contains the decarboxylase functional domain and the A gene product encodes an interaction domain which serves to stabilize the decarboxylase activity. The interaction domain may be present in the plant cells. Figure 54 and Table 17 showed test plants in group III all survived for 51 days except III-1, while a control nontransformed plant was destroyed 10 days after exposure to 4°C. Furthermore, six transformed plants survived for 51 days at 4°C, at which time they were moved back to the optimal temperature growth chamber.

The preliminary findings of this project would be strengthened by two additional experiments. First, the analysis of cell membrane fatty acid composition should be carried out to demonstrate the synthesis and incorporation of branched-chain fatty acids into the cell membranes of transgenic plants. Second, the ion leakage due to cold-mediated phase separation of the cell membrane should be compared in transgenic and untransformed plants to confirm that expression of the BCOADc gene(s) improves membrane fluidity.

12. Future experiments

Introduction of *B. subtilis* genes encoding BCOADc into tomato plants resulted in the successful transcription and translation of the bacterial enzyme. This project has provided data suggesting that the manipulation of straight-chain fatty acid synthesis in plants to produce branched-chain fatty acids increases tolerance to cold. The author hopes that this preliminary investigation of branched-chain fatty acid production in transgenic plants will encourage further study of this poorly understood biosynthetic pathway.

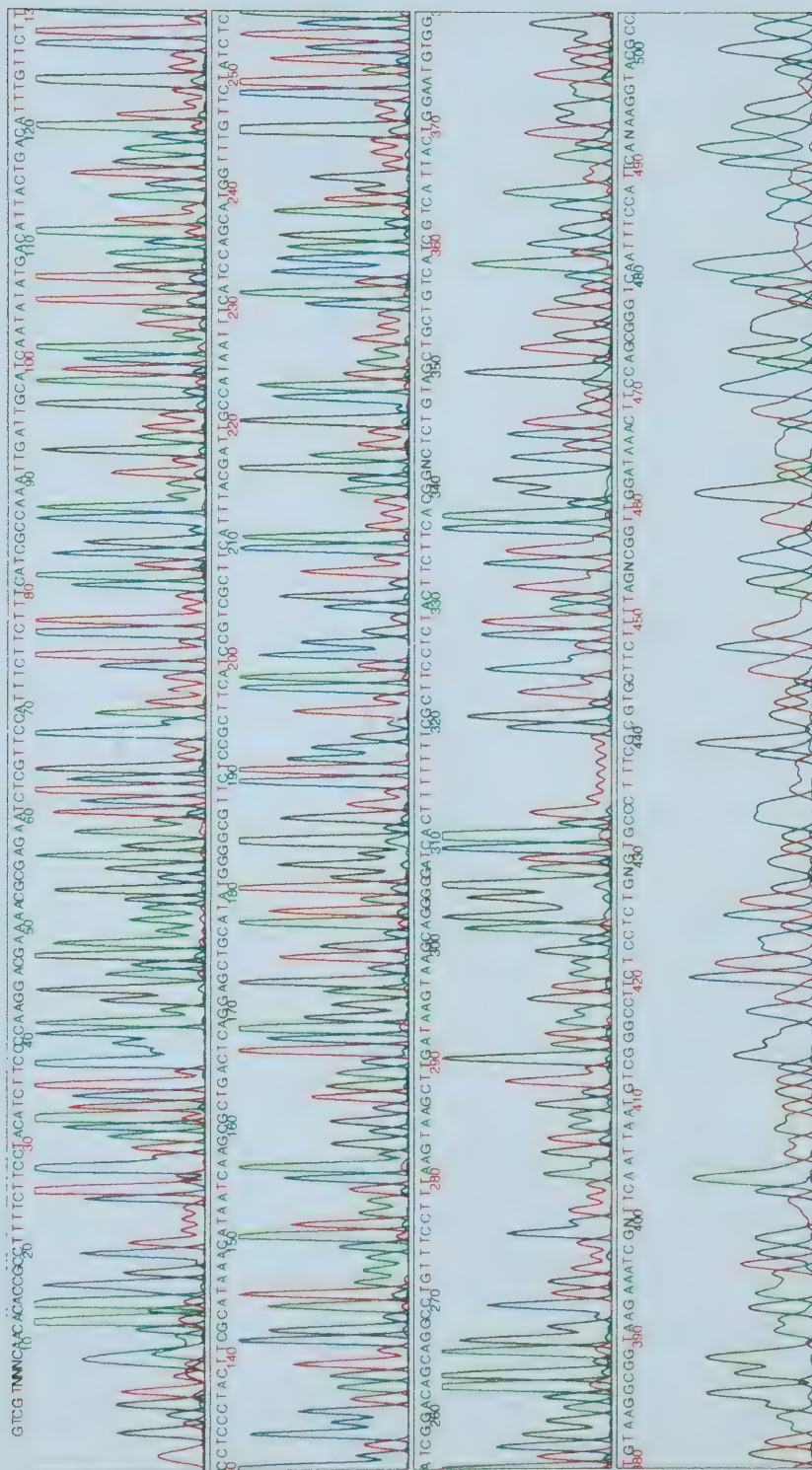
To continue this pioneering research, the following studies could be done:

- (1) Gas chromatography can be used to analyze the fatty acid composition of membrane lipids in transgenic plant tissues to confirm the presence of branched-chain fatty acids. In addition, do they replace saturated fatty acids more efficiently than unsaturated fatty acids?
- (2) Analysis of total proteins in plant organelles (i.e. chloroplasts and proplastids) by Western (immuno) blot analysis would help to determine whether the BCOADc becomes compartmentalized within the organelle containing the majority of the plant fatty acid biosynthetic enzymes or whether it acts in the cytoplasm. This would provide an important insight into the pathway for branched-chain fatty acid synthesis in plants.
- (3) The E2 polypeptide of the BCOADh enzyme complex cloned in the beginning of this project (Figure 11) could also be transferred to plants to study its effects on fatty acid synthesis.
- (4) Further insights into BCOADc function could be obtained by studying the *B. subtilis* CAC-11 mutation, which is deficient in this enzyme. Cloning and sequencing of the same operon in CAC-11 mutant will help us to determine the catalytic site of this enzyme, since the immunoblot study of this mutant demonstrated that the polypeptides of E1 α and E1 β are still produced from this mutant (Figure 51).
- (5) Other consequences of BCOADc gene expression in transgenic plants have yet to be

examined. These include possible effects on the absorption, penetration, and production of viruses in plant cells.

In addition, there may be other applications for transgenic tomato plants with branched-chain fatty acids in cell membranes. Delay of fruit ripening of tomato plants has economical significance. Although the introduction of branched-chain fatty acids into plant cell membranes did not appear to affect the growth morphology of transgenic plants in this project, theoretically branched-chain fatty acid incorporation into cell membranes could affect fruit ripening and senescence. Loss of cell membrane integrity, removal of permeability barriers, and increase in "free space" have long been suggested as major factors in ripening and senescence (Sacher, 1973). The role of oxidative breakdown of membrane lipids in plant senescence has attracted new interest in years (Leshem, 1981; Mazliak, 1983). It was reported that unsaturated fatty acids are used to synthesize components that inhibit the fruit ripening of tomato plants. If the incorporation of branched-chain fatty acids can replace unsaturated fatty acids at normal plant growth conditions, free unsaturated fatty acid pool will be enlarged, resulting in the potential delay of tomato fruit ripening.

Figure 56. Data of DNA sequencing from an automated sequencer ABI 373A.



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